

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV. 9-2001) TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER 58332-A-PCT-US/JPW/FHB U.S. APPLICATION NO. (if known, see 37 CFR 1.5 Not Yet Known 10/018677
INTERNATIONAL APPLICATION NO. PCT/US00/16594	INTERNATIONAL FILING DATE 15 June 2000	PRIORITY DATE CLAIMED 15 June 1999
TITLE OF INVENTION Generation of Antigen Specific T Suppressor Cells For Treatment of Rejection		
APPLICANT(S) FOR DO/EO/US The Trustees of Columbia University in the City of New York		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). (courtesy copy) b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (unsigned)</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>		
Items 11 to 20 below concern document(s) or information included:		
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Courtesy copy of Notice Informing Applicant of the Communication of the International Application to the Designated Offices, PCT Request, PCT Demand, Written Opinion, International Preliminary Examination Report, and Small Entity Declaration from U.S. parent application, 1 loose duplicate set of formal drawings (44pp), and an Express Mail Certificate of Mailing bearing Label No. EJ 700 080 215 US dated December 14, 2001.</p>		

14 DEC 2001

U.S. APPLICATION NO. (If known) 104018666 Not Yet Known	INTERNATIONAL APPLICATION NO. PCT/US00/16594	ATTORNEY'S DOCKET NUMBER 58332-A-PCT-US/JPW																									
<p><input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00</p>		CALCULATIONS PTO USE ONLY																									
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$ 710.00																									
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p>		\$ 0.00																									
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 20%;">CLAIMS</th> <th style="width: 20%;">NUMBER FILED</th> <th style="width: 20%;">NUMBER EXTRA</th> <th style="width: 20%;">RATE</th> <th style="width: 20%;">\$</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>56 - 20 =</td> <td>36</td> <td>x \$18.00</td> <td>\$ 648.00</td> </tr> <tr> <td>Independent claims</td> <td>17 - 3 =</td> <td>14</td> <td>x \$84.00</td> <td>\$ 1176.00</td> </tr> <tr> <td colspan="2">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td></td> <td>+ \$280.00</td> <td>\$ 0.00</td> </tr> <tr> <td colspan="2"></td> <td></td> <td></td> <td style="text-align: right;">\$ 2534.00</td> </tr> </tbody> </table>		CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	Total claims	56 - 20 =	36	x \$18.00	\$ 648.00	Independent claims	17 - 3 =	14	x \$84.00	\$ 1176.00	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$ 0.00					\$ 2534.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$																							
Total claims	56 - 20 =	36	x \$18.00	\$ 648.00																							
Independent claims	17 - 3 =	14	x \$84.00	\$ 1176.00																							
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$ 0.00																							
				\$ 2534.00																							
<p><input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.</p>		\$ 1267.00																									
<p style="text-align: right;">SUBTOTAL =</p>		\$ 1267.00																									
<p>Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).</p>		\$ 0.00																									
<p style="text-align: right;">TOTAL NATIONAL FEE =</p>		\$ 1267.00																									
<p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +</p>		\$ 0.00																									
<p style="text-align: right;">TOTAL FEES ENCLOSED =</p>		\$ 1267.00																									
<p style="text-align: right;">Amount to be refunded:</p>		\$																									
<p style="text-align: right;">charged:</p>		\$																									
<p>a. <input checked="" type="checkbox"/> A check in the amount of \$ 1267.00 to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>03-3125</u>. A duplicate copy of this sheet is enclosed.</p> <p>d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>																											
<p>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.</p>																											
<p>SEND ALL CORRESPONDENCE TO:</p> <p style="text-align: right;"></p> <p>John P. White, Esq. Reg. No. 28,678 Cooper & Dunham LLP 1185 Avenue of the Americas New York, NY 10036</p>																											
<p>SIGNATURE <u>John P. White</u></p> <p>NAME <u>John P. White</u></p> <p>28,678</p> <p>REGISTRATION NUMBER</p>																											

44/PRTS

10/018677

WO 00/76320

531 Rec'd PCT

14 SEP 2001

GENERATION OF ANTIGEN SPECIFIC T SUPPRESSOR CELLS FOR
TREATMENT OF REJECTION

5

This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/333,809, filed June 15, 1999, the contents of which is hereby incorporated by reference.

10

This invention was made with support under Grant Nos. 5-RO1-A125210-11, RO1A125210-10, and 5-RO1-A125210-12 from the National Institutes of Health. Accordingly, the United States Government has certain rights in the invention.

15

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

20

BACKGROUND OF THE INVENTION

25

Specific suppression of the host's immune response to donor HLA antigens remains the ultimate goal for clinical transplantation. In spite of considerable effort, however, allospecific human suppressor T cells (Ts) have been difficult to generate. The studies herein (first series of experiments) show that allospecific and xenospecific Ts can be raised by multiple priming of human T cells in mixed lymphocyte cultures (MLC). Ts derive from the CD8⁺CD28⁻ subset and recognize specifically the MHC class I antigens expressed by Antigen-Presenting Cells (APC) used for in vitro immunization. Allospecific Ts prevent the upregulation of B7 molecules on target APCs, interfering with the CD28-B7 interaction required for T helper (Th) activation. These findings provide a basis for the development of specific immunosuppressive therapy.

30

35

40

5 The induction of donor-specific tolerance remains the ultimate goal for clinical transplantation. Immunosuppressive treatments that have been developed so far act non-specifically, placing the recipient at increased risk for infections and malignancies.

10 Transplant tolerance has been induced in adult animals by inactivation or depletion of mature T lymphocytes prior to transplantation using cyclosporine (CsA) (1), total lymphoid irradiation (2,3), anti-lymphocyte serum (4), antibodies against CD4+ and CD8+ T cells (5), or donor-specific transfusions (6,7). Studies of peripheral graft tolerance have suggested the existence of an active mechanism of suppression which is donor-specific and can be transferred adoptively to secondary hosts (1, 7-10). However, there is still controversy concerning the phenotypic characteristics of these regulatory T cells and their MHC restriction, as both CD8⁺ and CD4⁺ T cells were reported to display suppressive activity (11). This controversy lead to the speculation that no distinctive T_s lineage actually exists. It has been suggested that suppression may result from antagonistic effects of (Th)2-type lymphokines (such as IL-4 and IL-10) on the response of T_{h1} cells (2,12), or from recognition by T_s of either idiotypic determinants of the TCR of alloreactive T cells or of MHC antigens expressed on stimulating cells (10,13). The generation of T_s lines has proven, however, to be a difficult task rendering the characterization of these cells hard to achieve.

20 The aim of the present study (first series of experiments) was to develop and characterize suppressor T cell lines which inhibit specifically the alloimmune response. This study established for the first time the existence of a population of CD8⁺CD28⁻ T_s which are allorestricted by HLA-class I antigens expressed by the cells used for priming. The mechanism of suppression is based on the capacity of T_s to prevent the upregulation of B7 molecules (CD80 and CD86) induced by Th on the stimulating APC. Allorestricted T_s can

be easily and reproducibly expanded in cultures facilitating the in vitro study of immunoregulatory networks and the development of new strategies for specific immunosuppression.

5 Evidence that T cells can down-regulate the immune response by producing or consuming certain cytokines or by lysing APCs or T helper cells has been provided in various systems. However, the generation and characterization of suppressor T cell lines have met with limited success. In the second series of experiments herein it is shown that xenospecific suppressor T cells can be generated by in vitro stimulation of human T cells with pig APCs. Similar to allospecific suppressors, these xenospecific suppressor T cells carry the CD8⁺CD28⁻ phenotype and react to MHC class I antigens expressed by the APCs used for priming. TCR spectratyping of T suppressor cells showed oligoclonal usage of TCR-V β families, indicating that xenostimulation of CD8⁺CD28⁻ T cells results in antigen-driven selection of a limited V β repertoire. Xenospecific T suppressor cells prevent the up-regulation of CD154 molecules on the membrane of T helper (Th) cells, inhibiting their ability to react against the immunizing MHC-class II xenoantigens. The mechanism of this suppression, therefore, appears to be blockade of CD154/CD40 interaction required for efficient costimulation of activated T cells.

20
25
30
35 The induction of regulatory T cells may offer an effective means for specific immunosuppression of autoimmune disease and allograft rejection. The existence of suppressor T cells has been previously documented, yet their mechanism of action remains poorly characterized. The third series of studies herein demonstrate that T suppressor (Ts) cell lines can be generated by in vitro immunization of human PBMCs, with synthetic peptides or soluble proteins coupled to beads. Such Ts cells express the CD8⁺CD28⁻ phenotype and show the following characteristics: a) antigen specificity and restriction by self MHC Class I molecules, b) limited TCR V beta gene usage, c) ability to inhibit antigen-specific, MHC

Class II restricted, Th proliferative responses, and d) capacity to downregulate and/or inhibit the upregulation by Th of CD40, CD80, and CD86 molecules on APCs. The inhibitory activity of Ts on Th proliferation requires the tripartite interaction between Th, Ts, and APCs and results from inefficient costimulation of Th.

Understanding the mechanism which underlies the induction of immunologic tolerance is crucial to the development of strategies for treatment of auto-immune diseases and allograft rejection. Although the concept that T suppressor cells (Ts) downregulate the immune response has long been accepted, the existence of a distinct population of lymphocytes that mediates suppression has not been convincingly demonstrated. In previous studies, human T cell lines (TCLs) were utilized to analyze the suppressive effects of CD8⁺ CD28⁻ T cells in allogeneic, peptide specific and xeno-specific responses. In each case, CD8⁺ CD28⁻ T cells inhibit proliferation of CD4⁺ T helper lymphocytes (Th) with cognate antigen specificity. These CD8⁺ CD28⁻ T cells display the critical functional characteristics of T suppressor cells. Similar to the induction of CD8⁺ cytotoxic T cells (Tc) by Th, this process depends on antigen presenting cells (APC) acting as a "bridge" between MHC-class I specific CD8⁺ and class II specific CD4⁺ T cells. A possible explanation of Ts-mediated suppression is their ability to modulate the function of APCs. The fourth series of studies herein show that CD8⁺CD28⁻ Ts directly inhibit the CD40 signaling pathway of APC by a contact-dependent mechanism that renders bridging APCs incapable of inducing CD4⁺ T cell activation. The effects of Ts on the functional state of APC supports the concept that the order in which Ts and Th cells interact with cognate APCs determines the functional outcome of immune responses.

5

10
15
20

25

30

35

SUMMARY OF THE INVENTION

This invention provides a method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.

This invention provides antigen specific allospecific human suppressor CD8+ CD28+ T cells produced by the method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28-

T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e).

This invention provides a method of generating xenospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a human subject; b) stimulating by multiple priming a human T cell line from the T cells obtained in step (a) with xenogeneic mammalian antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen; c) isolating primed human CD8+ T cells and human CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c); e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor CD8+CD28- T cells; f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the xenospecific human suppressor CD8+CD28- T cells.

This invention provides xenospecific human suppressor CD8+CD28+ T cells produced by the method of generating xenospecific human suppressor CD8+CD28- T cells which

comprises: a) obtaining peripheral blood T cells from a human subject; b) stimulating by multiple priming a human T cell line from the T cells obtained in step (a) with xenogeneic mammalian antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen; c) isolating primed human CD8+ T cells and human CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c); e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor CD8+CD28- T cells; and f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e).

20

This invention provides a method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with autologous antigen presenting cells (APCs) pulsed with an allopeptide, said allopeptide comprising an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences (motifs) and are recognized by the primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and autologous antigen presenting cells (APCs) expressing the same MHC class I and MHC class

10
15
20
25
30
35

II binding motifs as expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying allopeptide antigen specific human suppressor CD8+CD28- T cells; and f) expanding the allopeptide antigen specific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific human suppressor CD8+CD28- T cells.

This invention provides an antigen specific human suppressor CD8+CD28- T cells produced by the above-described method of generating the antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of determining whether a level of immunosuppresant therapy given to a subject undergoing the level immunosuppression therapy requires a reduction which comprises: a) obtaining a blood sample from the subject; and b) determining the presence of T suppressor cells present in the sample, the presence of T suppressor cells indicating that the subject requires the reduction of immunosuppresant therapy.

This invention provides a method of reducing the risk of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of reducing the level of

rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject, thereby preventing the rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of a xenograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject, thereby preventing the rejection of the xenograft in the subject.

-10-

This invention provides a method of preventing rejection of a xenograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating xenospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the xenograft in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,

thereby preventing autoimmune disease in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

This invention provides a vaccine comprising allospecific T suppressor cells stimulated by APCs expressing an MHC class I antigen and an MHC class II antigen which T suppressor cells suppress an interaction between CD4+ T helper cells and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the allospecific T suppressor cells.

-11-

This invention provides a vaccine comprising xenospecific T suppressor cells stimulated by APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen which xenospecific T suppressor cells suppress an interaction between CD4+ T helper cells and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the APCs used to stimulate the xenospecific T suppressor cells.

This invention provides a method of inducing anergic T helper cells which comprises: a) incubating antigen presenting cells (APC) with allospecific T suppressor cells (Ts); b) overexpressing in the APC mRNA which encodes at least one monocyte inhibitory receptor (MIR), in a mixture of cells comprising the APCs from step (a), wherein overexpression of MIR transmits negative inhibitory signals to recruit an inhibitory signaling molecule, tyrosine phosphatase SHP-1 such that the APC are rendered tolerogenic; and c) incubating the APCs from step (b) with T helper cells (Th) to induce Th anergy.

This invention provides a method of generating a tolerogenic antigen presenting cell (APC) which comprises: a) contacting the APC with Ts; and b) overexpressing mRNA which encodes an MIR in the APC, thereby generating a tolerogenic antigen presenting cell (APC).

This invention provides a method of reducing the level of rejection of an allograft tissue or organ in a subject who is a transplant recipient of the allograft tissue or organ which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), wherein the APC have been incubated with Ts prior to overexpression of MIR, thereby inducing Th anergy so as to prevent rejection of the tissue or organ allograft in the subject.

This invention provides a method of suppressing an autoimmune

-12-

disease in a subject which comprises: a) contacting antigen presenting cells (APC) of the subject with T suppressor cells (Ts) specific for the antigen which induces the autoimmune disease; and

5 b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) overexpressing monocyte inhibitory receptor (MIR) in antigen presenting cells (APC) of the subject, which APC present the antigen which induces the autoimmune disease ; and b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the allograft in the subject.

25 This invention provides a method of inducing tolerance to a xenograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the xenograft in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1D. Detection and characterization of CD8⁺CD28⁻ Ts in alloreactive TCLs. Fig. 1A. Reactivity of unseparated TCL, separated CD4⁺ and CD8⁺ T cell subsets, and mixtures of CD4⁺ and CD8⁺ T cells to the specific allostimulator was determined in blastogenesis assays. Results are expressed as mean c.p.m. of triplicate reactions. The SD of the mean was less than 10%. Fig. 1B. Dose-dependent suppression of CD4⁺ T cell alloreactivity to the specific stimulator in the presence of primed CD8⁺, CD8⁺CD28⁺, and CD8⁺CD28⁻ T cells. Mean c.p.m. in cultures without T_s was 28,470[±]1730. Fig. 1C. Flow-cytometry determination of target cell apoptosis. The percent of allogeneic APCs stained by Annexin V was determined after 4 and 24 hours of incubation with or without CD4⁺ and CD8⁺CD28⁻ T cells. Fig. 1D. Suppression of CD4⁺ T cell proliferation to specific stimulator by CD8⁺CD28⁻ T cells added to the cultures 0, 4, 8 and 16 hours after the initiation of the assay. Mean c.p.m. in cultures without T_s was 22,630[±]1860.

Figures 2A-2B. Antigenic specificity of CD8⁺CD28⁻ T suppressor cells. Fig. 2A. CD4⁺ T_n from TCL SS-anti-JL were tested in blastogenesis assay for reactivity against APC from the original stimulator JL and from donors sharing with JL HLA-class I and class II (PO) or only class II (ST). Separate cultures were stimulated with a mixture of APCs from two donors (GC sharing with JL class I and ST sharing class II). CD8⁺CD28⁻ T_s from TCL SS-anti JL were added at the initiation of the blastogenesis assay. Percent suppression was calculated from the ratio of c.p.m. in cultures containing mixtures of T_n and T_s and cultures containing only T_n. Mean c.p.m. in cultures without T_s was 33,212[±]2160, 27,630[±]1940, 28,430[±]2070 and 37,400[±]3450 when APCs from JL, PO, ST or mixtures of GC and ST were used as stimulators respectively. Fig. 2B. CD4⁺ T_n from TCL SS-anti-JL and

-14-

naive CD4⁺ T cells from SS were stimulated with APC from JL. Alloreactive CD8⁺CD28⁻ T cells from SS generated by priming against JL, GC (sharing with JL class I) or ST (sharing with JL class II) were added to these cultures. Percent suppression was calculated as above.

Figure 3. Suppression of xenoreactivity by CD8⁺CD28⁻ T cells primed to xenogeneic cells. TCL was generated by priming human T cells against pig PBMC. Reactivity of unseparated TCL, separated CD4⁺, CD8⁺, CD8⁺CD28⁻ T cell subsets and mixtures of CD4⁺ T cells with CD8⁺ or CD8⁺CD28⁻ population to pig PBMC was determined in 3-day blastogenesis assay.

Figures 4A-4H. Expression of CD80 and CD86 on allostimulatory APC. CD2⁻ depleted APCs from JL (the specific stimulator of TCL SS-anti-JL) were cultured for 24 hours without T cells (Figs. 4A and 4B), with CD4⁺ T cells from SS-anti-JL (Figs. 4C and 4D), purified CD8+ CD28- T cells from SS anti-JL (Figs. 4E and 4F) and both CD4⁺ and CD8⁺CD28⁻ T cells (Figs. 4G and 4H). Three-color flow cytometry was performed using mAbs anti-CD3 (for gating out the T cells), anti-CD80 and anti-CD86. Percent positive (%) and mean fluorescence intensity (MFI) of the positively staining population are indicated.

Figure 5. Prevention of suppression by mAb anti-CD28. CD4⁺ and CD8⁺CD28⁻ T cells were separated from TCL SS-anti-JL and tested alone or together for reactivity against APCs from JL in 3 day blastogenesis assays. MAbs anti-CD28 and anti-CTLA-4 (1 μ g/ml) were added to parallel cultures at the initiation of the assay. Percent suppression of CD4+ T cell proliferation induced by CD8⁺CD28⁻ T cells in the absence and in the presence of either mAb anti-CD28 or mAb anti-CTLA4 was calculated. Mean c.p.m. in T_s cultures without T_s were 32,510 \pm 2720.

Figures 6A-6B. Species specificity of CD8⁺CD28⁻ Ts cells. The

response of alloreactive (SA-anti-BM) (Fig. 6A) and xenoreactive (SA-anti-pig A) (Fig. 6B) human T cell lines against the specific stimulator was measured in a 3-day proliferation assay. Reactivity of the unseparated TCLs, separated CD4⁺ Th cells, and mixtures of CD4⁺ and CD8⁺CD28⁻ (Ts) cells from either TCL is illustrated. Results are expressed as mean counts/min of triplicate reactions. The SD of the mean is indicated.

Figures 7A-7B. The suppressive effect of xenoreactive CD8⁺CD28⁻ T cells does not involve idiotypic or MHC-restricted interactions between Ts cells and Th cells. The reactivity of xenoreactive T cell lines from two human donors (MN and AP; Figs. 7A and 7B) against APCs from the same pig (pig B) was tested in a 3-day blastogenesis assay. The response of the unfractionated TCLs, purified CD4⁺ cells, and mixtures of CD4⁺ and CD8⁺CD28⁻ cells from both lines to pig APCs is presented as mean counts/min of triplicate reactions. The SD of the mean is indicated.

Figures 8A-8B. Fig. 8A, Diffusion chamber experiments. Th cells from TCL CG anti-W were tested for reactivity to APC from the specific stimulator (strain W) on close contact with Ts cells (from the same TCL) or separated from Ts cells by a semipermeable membrane. Percent suppression of Th cells reactivity by Ts cells is indicated. Fig. 8B, Th cells from TCL ES-anti-W were tested for reactivity to APC from the specific stimulator (strain W) in the presence of autologous Ts cells and the indicated mAbs.

Figures 9A-9H. Cytokine profile of xenoreactive Th cells and Ts cells. Th cells and Ts cells from TCL CG-anti-pig Z were activated with PMA and ionomycin. Cells were treated with Brefeldin A, then fixed and stained with monoclonal antibodies specific for IL-2, IFN- γ , IL-4, and IL-10. Histograms obtained for the activated samples (solid line)

-16-

and resting control samples (dotted lines) are presented. The results are representative of three independent experiments.

Figures 10A-10C. Failure of Ts cells to induce killing of pig APCs or human xenoreactive Th cells. Fig. 10A, Pig APCs were incubated for 4 hours with Th cells, Ts cells, or both Th and Ts cells. The percent of early apoptotic (annexin V positive, PI negative) and late apoptotic/necrotic (annexin V positive, PI positive) pig APCs in cultures with and without xenoreactive human T cells was determined by flow cytometry. Camptothecin-treated APCs were used as positive controls for apoptosis. Fig. 10B, CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells from TCL ES-anti-Q were tested for their ability to kill PHA-stimulated target cells from strain Q in a ⁵¹Cr release assay. Results are expressed as percent lysis. Fig. 10C, Human Th cells were incubated with pig APCs in the presence or absence of Ts cells. The percent of CD4⁺ human T cells undergoing apoptosis was determined by staining with annexin V and PI. The percent of early apoptotic (annexin V positive, PI negative) and late apoptotic/necrotic (annexin V positive, PI positive) Th cells is shown. CD4⁺ Th cells treated with camptothecin served as positive controls.

Figures 11A-11D. Xenoreactive CD8⁺CD28⁻ T suppressor cells prevent up-regulation of CD40L expression on xenoreactive CD4⁺ T cells. Human CD4⁺ T cells were incubated for 6 hours without APCs (Fig. 11A), with APCs from the specific xenostimulator (pig W) (Fig. 11B), with APCs and Ts cells (Fig. 11C) or with control APCs from a pig (pig Z) that has different SLA class II antigens (Fig. 11D). CD154 expression on CD3⁺CD4⁺ human T cells was analyzed by flow cytometry. The percent CD154 positive T cells and the mean fluorescence intensity (MFI) are indicated. The results obtained with this TCL (CO-anti-pig W) are representative of data obtained from six TCLs.

-17-

Figure 12. $V\beta$ repertoire of unstimulated $CD8^+CD28^-$ T cells from individual MN and of xenoreactive Ts cells from TCL MN-anti-pig B expressed as relative intensity. To analyze spectratypes, relative intensity was calculated as the peak area corresponding to each $V\beta$ family divided by the sum of all peak areas.

Figures 13A-13D. $V\beta$ repertoire of unstimulated $CD8^+CD28^-$ T cells from individual ES (Fig. 13A) and of Ts cells from TCL ES-anti-pig Q (Fig. 13B), ES-anti-pig W (Fig. 13C), ES-anti-pig Z (Fig. 13D) expressed as relative intensity (histogram bars).

Figures 14A-14F. $V\beta$ spectratyping of Ts cells from TCL MN-anti-pig B. Only the families with positive signal are shown. x-axis, fragment lengths on base pairs; y-axis, fluorescence amplitude.

Figures 15A-15C. Distribution of $J\beta$ - $V\beta$ combination fragments in the $V\beta$ 9 (Fig. 15A), $V\beta$ 16 (Fig. 15B) and $V\beta$ 23 (Fig. 15C) families found in Ts cells from TCL ES-anti-pig Q. x-axis, fragment lengths on base pairs; y-axis, fluorescence amplitude.

Figure 16. Suppression of Th reactivity to rTT. $CD4^+$ Th cells from a TT-specific TCL were tested in a 3-day blastogenesis assay for reactivity to rTT-pulsed APCs. $CD8^+CD28^-$ Ts from the same TCL were added to the cultures at the initiation of the proliferation assay.

Figure 17. Suppression of Th reactivity to peptide Tat-DR4. $CD4^+$ Th cells and $CD8^+CD28^-$ Ts were purified from a Tat-DR4-peptide specific TCL and tested for reactivity to the synthetic peptide in cultures containing irradiated APCs. $CD8^+CD28^-$ Ts were added to Th cultures at the initiation of

the proliferation assay to measure suppressor activity.

5 **Figures 18A-18B.** Effect of anti-HLA class I mAbs on T cell reactivity to peptide Tat-DR4. CD4⁺ Th were tested for reactivity to peptide Tat-DR4 in medium without or with mAbs specific for the HLA-class I antigens expressed by the responder (Fig. 18A). Th and Ts were mixed together and tested for reactivity in the presence of MHC-class I specific mAbs (Fig. 18B).

10 **Figures 19A-1-19-C-5.** V β repertoire of CD4⁺ Th and CD8⁺CD28⁻ Ts primed to rTT. The relative intensity of TCR V β families expressed by CD8⁺CD28⁻ Ts after five weeks (Figs. 19A-19E) and six weeks in cultures (Figs. 19F-19J) and by CD4⁺ Th (Figs. 19K-19O) was calculated from the ratio between the peak area of each TCR and the sum of all area peaks.

15 20 **Figures 20A-20L.** CD8⁺CD28⁻ Ts prevent upregulation of CD40, CD80, and CD86 on antigen-loaded APCs. CD4⁺ Th and CD8⁺CD28⁻ Ts from a Tat-DR4 peptide specific T cell line were incubated alone or together with autologous APCs and antigen for 24 hours. The expression of CD40, CD80, and CD86 on autologous APCs (CD14⁺ and CD20⁺ cells) was analyzed by flow-cytometry. The percent positive APCs and the mean fluorescence intensity (MFI) are indicated.

25

30 **Figures 21A-21D.** Ts mediated suppression of Th activation and proliferation requires the presence of APCs. CD4⁺ Th and CD8⁺CD28⁻ Ts cells from the same TCL were activated alone or together either by mAb CD3 (Figs. 21A, 21B) or allogeneic APCs (Figs. 21C, 21D). Mouse IgG or autologous APCs were used as controls. Proliferation was determined in a three day assay (Figs. 21A, 21C), and CD40L expression on CD4⁺ T cells were analyzed after 6 h of culture (Figs. 21B, 21D). CPM of triplicate reactions are shown. SD to the mean was less than 10%. The results represent one of three independent

35

experiments.

Figure 22. Early recognition of APCs by Ts is required for suppression of Th proliferation. CD4+ Th cells isolated from a TCL were cultured with APCs used for priming for 3 days. CD8+CD28- Ts cells from the same TCL were added 0, 6, or 18 h after initiation of the culture. Mean CPM of triplicate cultures are shown. SD was less than 10% of the mean. T cell reactivity to self APCs was less than 2,000 cpm. The data are from one of four independent experiments.

Figures 23A1-23E-5. Early recognition of APCs by Ts is required for suppression of the expression of costimulatory molecules on APCs. APCs used for stimulation were cultures alone (Fig. 23A-1-23A-5) or with allospecific Th for 48 h (Fig. 23B-1-23B-5). Ts were added at the initiation of the cultures (Fig. 23C-1-23C-5), after 6 h (Fig. 23D-1-23D-5) or after 18 h (Fig. 23E-1-23E-5). Expression of costimulatory molecules on CD14+ and CD20+ cells were analyzed at the end of the incubation period. Percent of positive cells is indicated. The data are the results of one of three repeat experiments.

Figure 24. Exogenous IL-2 restores Th reactivity in the presence of Ts. CD4+ Th and CD8+CD28- Ts cells from the same TCL were activated alone or together with allogeneic APCs. rIL-2 (% units/ml) was added to parallel cultures at the initiation of the blastogenesis assay. CPM of triplicate reactions are shown. SD to the mean was less than 10%. The results represent one of three repeat experiments.

Figure 25. Ts suppress CD40-signaling in APC. The "suppressed" APC do not upregulate the expression of costimulatory molecules (CD80, CD86) and are, therefore, unable to induce and sustain the full program of Th activity.

Figure 26. HLA A, B and DR values and split equivalence.

-20-

Various HLA A loci, HLA B loci and HLA DR loci which may be used as antigens for priming T suppressor cells.

5

Figures 27A-27H. DRB Protein Sequences. Amino acid sequences of DRB proteins correspond to hypervariable regions of HLA-DR B1 antigens. These antigens may be used as allopeptides for priming T suppressor cells.

10
15
20
25
30
35

Figure 28. Detailed map of the swine major histocompatibility or swine leukocyte antigen (SLA) complex as compared to the human leukocyte antigen (HLA) complex. HLA-II ans SLA-II, HLA-II and SLA-III and Hla-I and SLA-I. (from J.K. Lunney and J.E. Butler, Immunogenetics, In The Genetics of the Pig, 1998, eds., M.F. Rothschild and A. Ruvinsky, CAB International.)

Figure 29. Amino acids sequences of SLA DRA alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

20

Figure 30. Amino acids sequences of SLA DRB alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

25

Figure 31. Amino acids sequences of SLA DQA alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

30

Figure 32. Amino acids sequences of SLA DQB alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

35

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.

Accordingly, the suppressor cells are obtained by a) isolating first the CD8+ T cells from the antigen-specific T cell lines; and b) isolating next the CD8+CD28- fraction from the CD8+ population above. Since only a small portion of suppressor T cells from step (d) is used for suppression analysis of step (e). Once suppression is detected in step (e) the cells isolated in step (d) may be expanded by techniques known to the skilled artisan, for example by weekly restimulation of the APCs used to stimulate the T cells of step (b) in culture medium containing recombinant human interleukin-2 (IL-2).

-22-

In an embodiment of the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells the MHC class I antigen is an HLA-A or HLA-B antigen expressed by the APC used for priming. Antigen specific suppressor cells can be generated by T cell priming against any of the existing HLA-A or HLA-B antigens of which there are more than two hundred such antigens. One of skill may select but is not limited to the HLA-A or HLA-B antigens from the group of HLA-A and HLA-B antigens listed in Figure 26.

In an embodiment of the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells the MHC class II antigen is an HLA antigen selected from the group consisting of HLA-DR, HLA-DQ and HLA-DP. One of skill in the art will recognize that there are hundreds of HLA class II antigens. For example HLA class II antigens may be but are not limited to DRB antigens which may be selected from but are not limited to the group of DRB proteins listed in Figure 27.

All APCs express two HLA-DR, HLA-DQ and two HLA-DP antigens. It is irrelevant which HLA-class II antigens are expressed by the APCs, in order to generate suppressor T cells. It is important, however, that the response of CD4+ T helper cells to allogeic APC can be inhibited only by CD8+ T suppressor cells which recognize the MHC class I antigens expressed by the same APC.

This invention provides an antigen specific allospecific human suppressor CD8+ CD28+ T cells produced by the method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating

primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.

This invention provides a method of generating xenospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a human subject; b) stimulating by multiple priming a human T cell line from the T cells obtained in step (a) with xenogeneic mammalian antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen; c) isolating primed human CD8+ T cells and human CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c); e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor CD8+CD28- T cells; f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the xenospecific human suppressor CD8+CD28- T cells.

-24-

Expansion techniques which may be used to culture cells in step (f) are well known to the ordinary skilled artisan. For example the expansion technique described above for the method of generating antigen specific allospecific human suppressor CD8+CD28- T cells may be used to expand xenospecific human suppressor CD8+CD28- T cells. In an embodiment of the above-described method of generating antigen specific xenospecific human suppressor CD8+CD28- T cells the xenogeneic antigen presenting cells (APCs) may be mammalian antigen presenting cells (APCs).

For example, the APCs may be pig APCs or primate APCs.

In an embodiment of the above-described method of generating xenospecific human suppressor CD8+CD28- T cells, the xenogeneic mammalian antigen presenting cells (APCs) are selected from pig or primate APCs. One of skill in the art will recognize that pig antigens may be selected from numerous SLA antigens class I antigens. The antigens may be selected from but are not limited to the group of SLA DRA, SLA-DRB, SLA-DRQ-A and SLA-DQB listed in Figures 29 through 32.

In an embodiment of the above-described method of generating xenospecific human suppressor CD8+CD28- T cells, the xenogeneic MHC class I antigen is selected from the group consisting of swine histocompatibility leukocyte antigen (SLA) class-I and MHC class II antigen is selected from the group consisting of swine histocompatibility leukocyte antigen (SLA) class-II. T cells specific for any SLA class I antigen or class II MHC antigen may be obtained by using said SLA class I or II antigens for priming. The SLA antigens may be expressed by the APCs used for priming. APCs of other mammals including all species of primates may be used according to the above-described method.

35

This invention provides a xenospecific human suppressor CD8+ CD28+ T cells produced by the above-described method of

generating xenospecific human suppressor CD8+CD28- T cells.

This invention provides a method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with autologous antigen presenting cells (APCs) pulsed with an allopeptide, said allopeptide comprising an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences (motifs) and are recognized by the primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and autologous antigen presenting cells (APCs) expressing the same MHC class I and MHC class II binding motifs as expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying allopeptide antigen specific human suppressor CD8+CD28- T cells; and f) expanding the allopeptide antigen specific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific human suppressor CD8+CD28- T cells. The identified human suppressor CD8+CD28- T cells are allospecific T suppressor cells. As discussed above any expansion method known to the skilled artisan may be used for expansion in culture of step (f).

In an embodiment of the above-described method of generating the antigen specific human suppressor CD8+CD28- T cells the allopeptide is a peptide antigen or a whole protein. For example the allopeptide may be selected from an allopeptide corresponding to hypervariable regions of HLA-DR B1 antigens which may be selected from but not limited to the HLA-DR B1 antigens listed in Figure 27.

This invention provides an antigen specific human suppressor CD8+CD28- T cells produced by the above-described method of generating the antigen specific human suppressor CD8+CD28- T cells.

5

This invention provides a method of determining whether a level of immunosupresant therapy given to a subject undergoing the level immunosuppression therapy requires a reduction which comprises: a) obtaining a blood sample from the subject; and b) determining the presence of T suppressor cells present in the sample, the presence of T suppressor cells indicating that the subject requires the reduction of immunosupresant therapy.

The presence of T suppressor cells in the sample may be determined in step (b) as follows: CD4+ T cells and CD8+ T cells are isolated from the recipient's (the subject undergoing the level immunosuppression therapy) blood. The CD8+CD28- T cell subset is isolated from the CD8+ population of T cells. II. Cultures are set up as follows: I. Recipient CD4+ Th plus donor APCs (depleted of CD2+ cells); Recipient CD8+CD28- T cells plus donor APCs (depleted of CD2+ cells); Recipient CD4+ Th plus CD8+CD28- T cells plus donor APCs. In control cultures donor APCs are replaced by APCs from a subject with different HLA-class I antigens. III. APCs are stained with mAb specific for CD19 and CD14 (PE, and with mAb specific for CD80 (FITC). IV. Suppression is considered to be present when the level of CD80 expression on donor APCs is 20% lower in cultures containing CD4+ Th and CD8+CD28- Ts than in cultures without Ts.

25

30

35

The presence of Ts in three consecutive samples of blood obtained at one month intervals indicates that the patient (recipient, i.e. subject undergoing the level immunosuppression therapy) is "accepting" the graft (transplant) and that immunosuppression can be tapered down, i.e. decreased.

In an embodiment of the above-described method of determining whether a level of immunosuppressant therapy given to a subject undergoing the level immuno suppression therapy requires a reduction the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a method of reducing the risk of rejection of an allograft in a subject undergoing immuno suppression therapy which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject.

In an embodiment of the above-described method of reducing the risk of rejection of an allograft in a subject undergoing immuno suppression therapy, the expansion in step (c) may be to produce an amount of approximately 10^7 T suppressor cells. One of skill is not limited to expanding the T suppressor cells to this amount of cells.

In another embodiment of the above-described method of reducing the risk of rejection of an allograft in a subject undergoing immuno suppression therapy the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immuno suppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immuno suppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells, thereby preventing

rejection of the tissue or organ transplant in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject, thereby preventing the rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of a xenograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject, thereby preventing the rejection of the xenograft in the subject.

In an embodiment of the above-described method of preventing rejection of a xenograft in a subject the T suppressor cells are suppressor CD8+CD28- T cells. The suppressor CD8+CD28- T cells have to be primed with APCs from the donor, i.e. to

specific xeno antigens before step (c), e.g. MHC class I antigens. CD8+CD28- T cells acquire T suppressor function only after priming with APCs expressing the donor's MHC class I antigens.

5

This invention provides a method of preventing rejection of a xenograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating xenospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the xenograft in the subject.

10

This invention provides a method of preventing autoimmune disease in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,

thereby preventing autoimmune disease in the subject.

20

In an embodiment of the above-described method of preventing autoimmune disease in a subject the expansion in step (c) may be to produce an amount of approximately 10^7 T suppressor cells. One of skill is not limited to expanding the T suppressor cells to this amount of cells.

25

In another embodiment of the above-described method of preventing autoimmune disease in a subject the T suppressor cells are suppressor CD8+CD28- T cells.

30

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

35

Suppressors CD8+CD28- T cells must be primed to acquire antigen specific function. The possible antigens which may

be used for priming includes all peptides known to elicit an autoimmune disease

5 This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

10 This invention provides a vaccine comprising allospecific T suppressor cells stimulated by APCs expressing an MHC class I antigen and an MHC class II antigen which T suppressor cells suppress an interaction between CD4+ T helper cells and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the allospecific T suppressor cells.

20 In an embodiment of the above-described vaccine comprising allospecific T suppressor cells the APCs are allogeneic APCs said APCs expressing an MHC class I antigen recognized by the T suppressor cells and an MHC class II antigen recognized by allogeneic CD4+ T helper cells. In another embodiment of the
25 above-described vaccine the APCs are APCs pulsed with an allopeptide, said allopeptide comprising an amino acid sequence having both MHC class I and MHC class II binding motifs wherein both motifs are recognized by the stimulated T suppressor cells. In an embodiment said allopeptide comprise an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences (motifs) and are recognized by the primed T cell line. In an embodiment of the above-
30 described vaccine the T suppressor cells are suppressor CD8+CD28- T cells.

35 This invention provides a vaccine comprising xenospecific T

5 suppressor cells stimulated by APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen which xenospecific T suppressor cells suppress an interaction between CD4+ T helper cells and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the APCs used to stimulate the xenospecific T suppressor cells.

10 In an embodiment of the above-described vaccine comprising xenospecific T suppressor cells wherein the T suppressor cells are suppressor CD8+CD28- T cells.

15 This invention provides a method of inducing anergic T helper cells which comprises: a) incubating antigen presenting cells (APC) with allospecific T suppressor cells (Ts); b) overexpressing in the APC mRNA which encodes at least one monocyte inhibitory receptor (MIR), in a mixture of cells comprising the APCs from step (a), wherein overexpression of MIR transmits negative inhibitory signals to recruit an inhibitory signaling molecule, tyrosine phosphatase SHP-1 such that the APC are rendered tolerogenic; and c) incubating the APCs from step (b) with T helper cells (Th) to induce Th anergy.

20 25 In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells.

30 35 In yet another embodiment of the above-described method, the Ts allopeptide are antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of generating a tolerogenic antigen presenting cell (APC) which comprises: a) contacting the APC with Ts; and b) overexpressing mRNA which encodes an

MIR in the APC, thereby generating a tolerogenic antigen presenting cell (APC) .

5 In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are antigen specific allospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells. In a still further embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

10
15 This invention provides a method of reducing the level of rejection of an allograft tissue or organ in a subject who is a transplant recipient of the allograft tissue or organ which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), wherein the APC have been incubated with Ts prior to overexpression of MIR, thereby inducing Th anergy so as to prevent rejection of the tissue or organ allograft in the subject.

20
25 In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In yet another embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

30
35 This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) contacting antigen presenting cells (APC) of the subject with T suppressor cells

-33-

(Ts) specific for the antigen which induces the autoimmune disease; and

b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

5

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells.

15 In another embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

20

This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) overexpressing monocyte inhibitory receptor (MIR) in antigen presenting cells (APC) of the subject, which APC present the antigen which induces the autoimmune disease and are genetically engineered to overexpress MIR; and b) administering to the subject the APC of step(a),
25 thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

25

30

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In still another embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells. In an
35

-34-

embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

5 This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the allograft in the subject.

This invention provides a method of inducing tolerance to a xenograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the xenograft in the subject.

20 This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

25

METHODS AND RESULTS

First Series of Experiments

30 Abbreviations used herein are: CsA - Cyclosporine; TCL - T cell line; Th - T helper cell; Ts - T suppressor cells
TNF - tumor necrosis factor; PIN - Perforin-induced necrosis;
PBMC - peripheral blood monoclear cells; PE - Phycoerythrin;
MLR - Mixed lymphocyte reaction; APC - antigen-presenting cell.

35

METHODS

5 **HLA Typing.** Lymphocytes were typed for HLA class I and class II antigens by conventional serology. The class II genotype of the cells was determined by genomic typing of in vitro amplified DNA with sequence-specific oligonucleotide probes for DRB1, DQA1 and DQB1.

10 **Generation of alloreactive T cell lines (TCL).** Peripheral Blood mononuclear cells (PBMC) from healthy blood volunteers were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding PBMCs (1×10^6 /ml) were stimulated in 24-well plates with irradiated (1600 rad) APCs (0.5×10^6 /ml) obtained from allogeneic PBMC by depletion of CD2⁺ cells. Cells were co-cultured for 7 days in complete medium (RPMI 1640 supplemented with 10% human serum, 2mM L-glutamine and 50g/ml gentamicin) (GIBCO, NY). After 7 days responding cells were collected, washed and rechallenged with the original stimulating cells. Three days later rIL-2 (Boehringer Mannheim, IN) was added (10 μ g/ml) and the cultures were expanded for an additional 4 days. Blastogenesis assays were performed on day 14. The HLA phenotypes of the responders and stimulators used for generating the seven different T cell lines used in these experiments are shown in Table 1. The xenoreactive TCL were generated by the same method using irradiated pig PBMC as stimulating cells.

Table 1 HLA Phenotypes of the Blood Donors

Donor ID	A	B	DR	DQA	DQB
TR	1, 2	7, 37	0701, 1501	0102, 0201	0201, 0602
RV	31, 33	35, 49	0701, 1501	0102, 0201	0201, 0602
NB	3, 24	27	0101, 0401	0101, 0301	0501, 0302
SS	26	35, 52	0402, 1502	0103, 0103	0302, 0601
JL	3, 29	44, 57	0701, 0701	0201, 0201	0201, 0201
GC	3, 74	45, 49	0801, 0801	0401, 0401	0402, 0402
ST	2, 24	13, 38	0701, 1301	0201, 0103	0201, 0603
PO	29, 31	44, 61	0701, 1101	0201, 0501	0201, 0301
LZ	2, 11	39, 67	1101, 1201	0501, 0501	0301, 0301

Cell separation and culture. The CD4⁺ and CD8⁺ T cell subsets were isolated from PBMC by negative selection using Dynal CD4⁺ and CD8⁺ magnetic beads (Dynal, NY). Goat-anti-mouse Dynal beads were coupled with mAb anti-CD28 (Becton Dickinson, CA) according to the manufacturer's instructions. To separate CD28⁺ and CD28⁻ T cells from CD8⁺ T cell suspensions, isolated CD8⁺ T cells (1×10^7 /ml) were incubated with 4×10^7 CD28 beads for 20 min at 4°C. The suspension was then placed on a magnetic particle concentrator for 2-3 minutes. The unbound cells (CD8⁺CD28⁻) were transferred to another tube and washed three times in complete medium. The bound CD8⁺CD28⁺ T cell population was detached from the beads by overnight incubation at 37°C. Cells were collected, washed and resuspended in complete culture medium. Cytofluorographic analysis showed that the purity of CD4⁺, CD8⁺ and CD8⁺CD28⁺ suspensions was >96%. The CD8⁺CD28⁻ population contained less than 7% CD28⁺ T cells.

Flow Cytometry. T cell subsets were defined using mAb CD4-PerCP, CD8-FITC, CD28-phycoerythrin from Becton Dickinson Immunocytometry System, CA. Cell suspensions were phenotyped prior to use in blastogenesis assays using a FACScan flow cytometer instrument (BDIS) equipped with a 15mm Argon Laser.

To study the effect of T_h and T_s on the expression of B7 molecules on allogeneic APC, CD4⁺ and CD8⁺ T cells were isolated from alloreactive TCL by positive selection using CD4 and CD8 magnetic beads. The CD8⁺CD28⁻ subset was obtained from the CD8 population by negative selection using beads coupled with anti-CD28 mAB. T_h cells were cultured with APC from the specific stimulator at a 5:1 ratio. T_s were cultured with the APC at a 1:1 ratio. In mixed cultures containing T_h, T_s, and allogeneic APC the ratio was 5:1:1. APC to which no T cells were added served as a control. After 24 hours of incubation cells were stained with saturating amounts of mAbs recognizing CD3-PerCP, CD80-PE (Becton Dickinson) and CD86-FITC (Pharmingen, CA). CD3⁺ T cells were gated out, and the remaining cells were analysed

using CellQuest software on a 650 Apple Macintosh Computer. Five parameter analysis (forward scatter, side scatter and 3 fluorescence channels) were used for list mode data analysis. FL3 channel was used as fluorescence trigger, FL1 and FL2 as analysis parameters. Mouse IgG (γ 1 and γ 2) reagents were used as isotype controls for non-specific binding of test reagents and as markers for delineating the positive and negative populations. CaliBrite flow cytometer beads (Becton Dickinson) and FACSCcomp program were used for calibration of the cytometer.

Flow Cytometry Analysis of Apoptosis. The capacity of alloreactive CD8 $^{+}$ CD28 $^{-}$ T cells to induce apoptosis of CD2-depleted allogeneic APC was tested by flow cytometry using annexin V as a marker for apoptotic cells. APCs were incubated for 4 hrs or 24 hrs at 37° with T_h, T_s, and mixtures of T_h and T_s. The ratio between T cells and APC was 5:1. Cells were stained with mABs recognizing CD20-PE or CD4-PE (Becton Dickinson). After 15 min. of incubation cells were washed and stained with annexin V-FITC and propidium iodide according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Log FL2 (PE) versus Side scatter parameters were used to gate on CD20 $^{+}$ or CD4 $^{+}$ cells. Log FL1 (FITC) versus FL3 (PI) dot plot of the gated cells were used for cell apoptosis and necrosis analysis.

Proliferation Assays. Unseparated responding T cells were tested for blastogenesis at a concentration of 5x10 4 /well. T cell subsets sorted from the same cultures (CD4 $^{+}$ T, CD8 $^{+}$ T, CD8 $^{+}$ CD28 $^{+}$ T, or CD8 $^{+}$ CD28 $^{-}$ T) were tested at a concentration that corresponded to their frequency in the unseparated population as determined by flow cytometry analysis of the TCL and of each population sorted. In experiments which required no comparison between unseparated TCL and T cell subsets, CD4 $^{+}$ and CD8 $^{+}$ T were tested at 2.5x10 4 cells/well and CD8 $^{+}$ CD28 $^{-}$ T cells were tested at 1.25x10 4 cells/well. Cell concentrations were adjusted after establishing the

5 purity of the fraction by flow cytometry. In all blastogenesis assays the concentration of APC used for stimulation was 5×10^4 /well. After 48 hr of incubation, the cultures were pulsed with [³H] Thymidine and harvested 18 hr later. [³H] Thymidine incorporation was determined by scintillation spectrometry in an LK Betaplate counter. Mean cpm of the triplicate cultures and the standard deviation to the mean were calculated.

10 The percent suppression was calculated as follows:

% suppression = $[1 - (\text{c.p.m. in mixed cultures of activated CD4}^+ T_n \text{ and } CD8^+CD28^- T_s) / (\text{c.p.m. in cultures with activated CD4}^+ T_n)] \times 100$

15 The effect of mAb anti-CD28 (clone 37407.11 from R & D Systems, Minneapolis, Mn) and anti-CTLA4 (clone BN 13 from Coulter, Miami, Fl) on the suppressor effect was tested by adding the mAb to the cultures (1 μ g/ml) at the initiation of 20 the blastogenesis assay.

25 **Statistical Analysis.** Statistical analysis of the results was performed using BMDP statistical software. Analysis of variance to assess significance of group differences (ANOVA) followed by Tukey's method for multiple comparison was applied to flow cytometry results. Correlation coefficients were obtained using Linear Regression Analysis.

Results

30 Suppression of alloreactivity is mediated by CD8⁺CD28⁻ T cells and is dose-dependent. TCL were generated by stimulating PBMC from HLA unrelated individuals with irradiated APCs from blood donors mismatched from the responders for both HLA-class I and class II antigen. The 35 HLA phenotypes of the blood donors used in this study are shown in Table 1. Although all TCL showed strong reactivity after primary and secondary stimulation, they

displayed low reactivity when challenged for the third time in 3-day blastogenesis assays.

However, strong reactivity of CD4⁺ T cells to the specific stimulator was restored when CD8⁺ T cells were depleted from the TCL. CD8⁺ T cells from the same culture showed little blastogenic response against the stimulator. When the cultures were reconstituted by mixing together the CD4⁺ and CD8⁻ subsets of cells, at the original ratio, the response of CD4⁺ Th to the allogeneic priming cells was inhibited (Fig. 1A). The percent inhibition by CD8⁺ T_s from different TCL ranged from 50 to >90% (mean 76%±23%). These experiments indicate that the CD8⁺ fraction of the TCL contains a population of Ts which suppress the proliferative response of CD4⁺ T_s against the specific stimulator.

To characterize the population of CD8⁺ T cells which mediate suppression, the CD8⁺CD28⁺ and CD8⁺CD28⁻ populations from an alloreactive TCL (TCL-LZ anti-NB) were separated and tested for their capacity to inhibit the proliferative response of CD4⁺ T cells from the same TCL against the specific stimulator (NB). The CD8⁺ CD28⁻ T cells exhibited dose-dependent suppression of the CD4⁺ T cell response to the specific allostimulator, while the CD8⁺CD28⁺ T cells had no inhibitory effect (Fig. 1B). Naive CD8⁺ T cells from the peripheral blood of the same responder had no inhibitory effect on the reactivity of CD4 T cells from this TCL (data not shown).

Since the kinetics of proliferative responses can shift under certain culture conditions the proliferative response of Th cells to allogeneic APC was measured in the presence or absence of T_s cells, by harvesting the cultures after 24, 48, 72, 96, and 120 hours of incubation. The data in Table 2 show that suppression of Th proliferation became detectable after 48 hours. The magnitude of the suppressor effect increased over the next 3 days of incubation. Because the peak of Th

blastogenesis occurred on day 3 all suppressor assays were harvested after 72 hours of incubation.

To establish whether killing of stimulating cells by allo-activated CD8+ T cells contributes to the suppressive effect, the ability of separated CD8⁺CD28⁺ and CD8 CD28⁻ T cells to lyse PHA-activated target cells from the specific stimulator was tested. ⁵¹Cr release assays showed that the cytotoxic activity resided entirely in the CD8⁺ CD28⁺ T cell population (data not shown).

Since lymphocyte-mediated cytotoxicity maybe caused not only by perforin induced necrosis (PIN) of the target but also by PIN/granzyme induced apoptosis (14) the capacity of T_s to cause apoptosis of allogeneic target cells was tested. No evidence of T_s -induced apoptosis was found. The percent apoptotic APC was similar in cultures to which T_s cells were added and in cultures containing only APC or T_h and APC (Fig. 1C). Also T_s did not cause apoptosis of T_h as the percentage of annexin V positive T_h was the same in cultures with or without T_s (data not shown). Hence the suppressive activity exhibited by allostimulated CD8⁺CD28⁺ T cells on the proliferative response of CD4⁺ T cells is not caused by killing of the stimulating cell targets or by killing of Th cells.

Table 2
Kinetics of the responses of T_h and T_s cells

Incubation time (h)	(^3H) Thymidine incorporation (mean c.p.m. \pm SD)				$T_h + T_s$	
	T_h		T_s			
	LZ	NB	LZ	NB	LZ	NB
24	14,190 \pm 986	17,840 \pm 1532	2315 \pm 204	3796 \pm 284	13,806 \pm 1067	16,396 \pm 1353
48	3081 \pm 274	31,783 \pm 2654	454 \pm 29	3529 \pm 310	4,143 \pm 342	16,878 \pm 1625
72	599 \pm 57	31,673 \pm 2015	92 \pm 8	970 \pm 67	491 \pm 42	7606 \pm 548
96	1610 \pm 135	23,372 \pm 1763	57 \pm 4	155 \pm 14	162 \pm 12	780 \pm 67
120	145 \pm 14	15,940 \pm 1032	50 \pm 3	84 \pm 7	96 \pm 7	820 \pm 75

CD4^+ T_h cells from TCL (LZ anti-NB) were tested for reactivity to specific target cells in the absence or presence of $\text{CD8}'\text{CD28}'$ T_s cells derived from the same TCL. The cultures were harvested at 24, 48, 72, 96, and 120h after initiation.

-43-

Suppression of CD4⁺ T cell alloreactivity by allostimulated CD8⁺CD28⁻ T cells is an early event. To investigate the kinetics of the suppressive effect, CD8⁺ CD28⁻ T cells were isolated from an alloreactive TCL (TR anti-NB) and added to CD4⁺ T cells from the same TCL at the initiation of the blastogenesis assay or after 4, 8 or 16 hours. Results obtained on day 3 showed that suppression was highest (90%) when CD8⁺CD28⁻ T cells were added at the initiation of the cultures (Fig. 1D). When Ts were added 4 or 8 hours after exposure of CD4⁺ T cells to the specific stimulator, the inhibitory activity decreased to 50 and 20 percent, respectively. No inhibitory effect was observed when the addition of Ts was delayed by 16 hours after activation of CD4⁺ T cells. The correlation between the time when Ts were added and their inhibitory effect was statistically significant ($r= 0.953$, $p< 0.05$). This indicates that suppression of specific reactivity of Th cells by activated CD8⁺CD28⁻ T cells is an early event.

In an attempt to determine which surface molecule(s) and lymphokines may play a role in suppression we performed blocking experiments in which various mAbs were added to the cultures at the initiation of the blastogenesis assay. Antibodies to IL-4, IL-10 and (TNF)- β did not block the suppressive activity of CD8⁺CD28⁻ T cells, indicating that these cytokines are not the mediators of the inhibitory effect. The addition of anti-HLA-class I mAb to the cultures, however, reduced the amount of suppression, indicating that allorecognition of HLA class I antigens by Ts is required for suppression to occur (data not shown).

Antigenic specificity of alloactivated CD8⁺CD28⁻ T_s. To determine the nature of the HLA-antigens recognized by CD8⁺CD28⁻ T_s, APC sharing with the original priming cells HLA-class II (required for responder CD4⁺ T cell activation), or both HLA class I and class II antigens were used as stimulators in suppressors assays. One of the six

-44-

representative experiments is described below.

Ts obtained from TCL SS-anti-JL inhibited by 51% the response of separated CD4⁺ T cells to the original stimulator (JL). Reactivity against other APC (PO) sharing both HLA-class I and class II antigens was equally suppressed, while the response to APC (ST) sharing only class II antigens with the specific stimulator was not affected (Fig. 2A). Hence, CD8⁺CD28⁻ Ts recognize specifically HLA-class I antigens expressed by the APC used for in vitro immunization.

To establish whether APCs must co-express the target antigens recognized by CD4⁺ Th and CD8⁺CD28⁻ Ts for suppression to occur, mixtures of two APC, sharing with the original priming cells either class II (ST) or class I (GC) antigens (but not both) were used for stimulation. There was no inhibition of CD4⁺ Th reactivity by CD8⁺ CD28⁻ Ts in these blastogenesis assays, indicating that suppression requires cell-to-cell interaction between CD4⁺ Th, CD8⁺ Ts and allogeneic APC expressing the class I and class II antigens against which the T cells were primed (Fig. 2A).

To further substantiate the conclusion that CD8⁺CD28⁻ Ts recognize HLA-class I antigens on allogeneic APC, CD4⁺ T cells from TCL SS-anti-JL were mixed with autologous CD8⁺CD28⁻ Ts from other TCLs which had been primed against APC sharing with the original stimulator (JL) either HLA-class I (GC) or class-II (ST) antigens (Fig. 2b). Suppression was induced by CD8⁺CD28⁻ T cells (TCL SS-anti-GC) primed against shared HLA-class I antigen(s) (GC). CD8⁺CD28⁻ T cells from a TCL (TCL SS anti-ST) primed against shared HLA-DR, but different class I alloantigens (ST), had no suppressive effect. Hence, Ts generated by allostimulation are allorestRICTed by HLA-class I antigens.

35

To determine whether CD8⁺CD28⁻ Ts can also inhibit the recognition phase of the MLC response and whether such an

effect is allo-MHC-class I restricted, alloactivated CD8⁺CD28⁻ T cells were added to a primary MLC at the initiation of the cultures. For this MLC, naive CD4⁺ T cells obtained from peripheral blood (SS) were tested as responders against one stimulator (JL). Ts from the three TCL used in the experiment described above were tested for inhibitory activity. Only Ts which had been activated against the allogeneic stimulator used for the primary MLC (JL) or against APC sharing with JL an HLA-class I antigen (GC) were able to inhibit the primary MLC. CD8⁺CD28⁻ T cells from a TCL primed against stimulating cells sharing with JL HLA-class II but not class I antigens (ST) had no suppressor effect (Fig. 2B). These data indicate that alloactivated CD8⁺CD28⁻ T cells which recognize HLA-class I antigens specifically suppress the activation of CD4⁺ T cells via the direct recognition pathway.

Lack of MHC restriction of T_h-T_s interaction. The interaction between alloreactive CD4⁺ T_h and CD8⁺CD28⁻ T_s may require T cell recognition of peptide(s) presented by self HLA-class I or class II antigens expressed by the responding or suppressing T cell population. To examine this possibility we generated alloreactive TCLs by priming T cells from three individuals (TR, RV and LZ) against the same allogeneic APC (NB). These three responders shared with each other either class I or class II antigens: RV shared with TR HLA-DR and DQ but not HLA-A, B; LZ shared with TR only HLA-A2. CD4⁺ T cells from one of these TCLs (TR anti-NB) were next tested in 3 day blastogenesis assays for reactivity against APC from the specific stimulator. The proliferative response of CD4⁺ T cells was inhibited with equal efficiency by autologous and allogeneic CD8⁺CD28⁻ T cells which had been primed to the same stimulator. Thus, the regulatory effect of CD8⁺CD28⁻ T cells on CD4⁺ responding T cells is not restricted either by the HLA-class I or class II antigens which they express (Table 3).

5 **Suppression of xenoreactivity by CD8⁺ CD28⁻ T cells.** The finding that the direct recognition pathway of alloreactivity can be suppressed specifically by activated CD8⁺CD28⁻ T cells has important implications for specific inhibition of allograft immunity. To determine whether in vitro educated T cells can also inhibit direct recognition of xenogeneic target cells, we generated xenoreactive TCLs by priming human PBMC with irradiated PBMC from a pig. Responding cells were primed on day 0 and restimulated on day 7 with pig PBMC. IL-2 was added on day 10, and the cultures were tested on day 14 for reactivity against pig PBMC. While the non-fractionated TCL showed low blastogenic responses, the purified CD4⁺ subset showed strong reactivity to pig stimulating cells in 3-day blastogenesis assays. Neither unseparated CD8⁺ T cells or separated CD8⁺CD28⁻ T cells proliferated in response to the priming cells. When added to CD4⁺ responding cells at the beginning of the assay both CD8⁺ and CD8⁺CD28⁻ T cells strongly inhibited the proliferative response (Fig. 3). These results were confirmed in a large series of experiments. Experiments using inbred strains of swine showed that T_s were xenorestricted by MHC-class I antigens (see Third Series of Experiments).

Table 3
Lack of MHC Restriction of T Helper-Suppressor Cell Interactions

CD4 ⁺ T responding cells	CD+CD28-T _s		[³ H] Thymidine Incorporation (mean cpm)
	Stimulators		
	TR	NB	
TCL TR anti-NB			1,159±80
TCL TR anti-NB	TCL TR anti-NB		689±50
TCL TR anti-NB	TCL RV ^a anti-NB		4,199±370
TCL TR anti-NB	TCL LZ ^b anti-NB		4,271±280
			24,454±1960
			611±40
			1,179±90
			785±80

^aSharing with TR HLA-DR, and DQ but not HLA-A and B.

^bSharing with TR only HLA-A2.
 CD4⁺ T cells from an alloreactive TCL (TR anti-NB) were tested for specific alloreactivity in the presence of CD8⁺ CD28⁺ T cells from the same TCL or from other TCLs which have been generated by priming PBMC from unrelated blood donors against the same allogeneic stimulator.

These data indicate that human CD8⁺ CD28⁻ T cells can be educated in vitro to suppress the response of CD4⁺ T cells against xenogeneic target cells.

5 **Expression of CD80 and CD86 on stimulatory APC in the presence of T_s.** Since inhibition of alloreactivity occurred only when the stimulating APCs were recognized by both T_s and T_h, we examined the possibility that T_s interfere with the expression of costimulatory molecules. Interaction of CD28 on T cells with B7 molecules (CD80 and CD86) on APCs is the most important costimulatory pathway for the response to alloantigens (15,16). CD80 and 86 are absent on resting B and T cells, but are induced after activation. While CD86 is constitutively present on resting monocytes, CD80 is expressed only after stimulation with IFN- γ (17). To determine whether T_s alter the pattern of B7 expression on allogeneic APC, we cultured T_h and/or T_s from five different individuals with CD2-depleted APC from the specific allogeneic stimulator. Figure 4 illustrates the results of one of these experiments. Analysis of CD80 and CD86 expression on stimulating APC, showed that after 24 hours the level of expression increased significantly in the presence of alloreactive CD4⁺ Th (Figs. 4C and 4D) compared with the level of expression in the absence of T cells (Figs. 4A and 4B) or in the presence of CD8⁺CD28⁻ T_s (Figs. 4E and 4F). APC from parallel cultures containing both T_h and T_s displayed much lower levels of CD80 and CD86 expression (Figs. 4G and 4H) than APC cultured only with T_h. This indicates that allospecific CD8⁺CD28⁻ T_s interfere with the upregulation of CD80 and CD86 expression induced on stimulating APCs by alloreactive CD4⁺ T_h.

30 To analyze the statistical significance of the decreased B7 expression in cultures containing T_s, the results obtained in the five different experiments were grouped together. Comparison of the mean percentage of target cells expressing B7 molecules in cultures containing only T_h and in cultures

with both T_h and T_s showed that the decrease in the upregulation of B7 expression in the presence of T_s was statistically significant (Table 4).

5 The impaired upregulation of CD80 and CD86 on stimulating APC may prevent the efficient costimulation of T_h in the presence of T_s . Alternatively, this may be the consequence rather than the cause of T_h inhibition by T_s . To explore these possibilities we tested the effects of mAbs anti-CD28 and anti-CTLA-4 on the reactivity of $CD4^+ T_h$ from a TCL (SS anti-JL) to the specific stimulator (JL) in the presence and absence of $CD8^+CD28^- T_s$. MAb anti-CTLA-4 did not affect T_s -mediated inhibition of the Th proliferative response. In contrast, ligation of CD28 by use of mAb anti-CD28 restored the ability of $CD4^+ T_h$ to respond to the specific stimulator in the presence of $CD8^+CD28^- T_s$ (Fig. 5). This result is consistent with the notion that suppression is caused by defective costimulation.

10

15

Table 4

Effect of T_s cells on Th induced up-regulation of B7 molecules on APC

Percentage of B7 ⁺ target cells			
	APC	T _h +T _s +APC	T _h +APC
CD80	12.4 ± 4.6 ^b	20.4 ± 4.0 ^b	47.2 ± 11.8
CD86	18.6 ± 12.4 ^b	35.8 ± 15.5 ^c	66.4 ± 7.1

^aP value for differences between all groups was computed by ANOVA.

^b The difference between B7 expression on targets incubated with T_h versus targets incubated with T_s, T_s plus T_h or medium was significant ($P < 0.01$).

^c The difference between CD86 expression on targets incubated with T_h only versus targets cultured with both T_h and T_s was significant ($P < 0.05$).

DISCUSSION

The present study demonstrates that allospecific and xenospecific human Ts can be generated and expanded in vitro by multiple priming of PBMCs with allogeneic or xenogeneic stimulator cells. Alloreactive Ts derive from the CD8⁺CD28⁻ population of T lymphocytes and recognize specifically the MHC-class I antigens expressed by the allogeneic APC used for in vitro immunization. Suppression of Th alloreactivity occurred only when the stimulatory APC co-expressed MHC class II antigens recognized by the Th to be suppressed and MHC-class I antigens recognized by the suppressor T cell population. Thus, CD4⁺ T_h reactivity was not inhibited by CD8⁺ CD28⁻ T_s when the cells were stimulated in blastogenesis assay with mixtures of two allogeneic APCs sharing with the original priming cells either MHC class I or class II antigens. Suppression of alloreactive T_h, therefore, requires cell-to-cell interaction between CD4⁺ Th, CD8⁺ CD28⁻ T_s and allogeneic APC expressing the class I and class II antigens against which the T cells were primed. Since no suppression occurred when mixtures of allogeneic APC were used, it is unlikely that competition between T_h and T_s for the surface of the APC or locally produced IL-2 is the mechanism underlying suppression (18). The role of other lymphokines such as IL-4, IL-10 and TNF- β can also be excluded as antibodies to these lymphokines did not block suppression. Suppression was an early event as it did not occur when the addition of T_s was delayed by more than 8 hours after T_h stimulation.

The need for cell-to-cell interaction between T_h, T_s, and APC, suggests that T_s may act by inhibiting costimulatory signals delivered by the APCs used for priming. This possibility is strongly supported by our finding that the addition of T_s to the cultures inhibited Th-mediated upregulation of CD80/CD86 expression on stimulating APC. Suppression was first detected after 48 hours of co-culturing Th and Ts consistent with the finding that CD80/CD86 down

modulation was seen at 24 hours. Therefore, it appears that T_s induce early changes in target APC, by a yet unknown mechanism, which interfere with the upregulation of B7 molecules required for T_h co-stimulation.

5

The B7 family has been shown to play a critical role in providing T cell costimulation which is required for the induction of maximal proliferation and cytokine production (15-17). It has been shown that T cells are sensitive to quantitative changes in the molecular interactions that contribute to antigen recognition such as those transmitted through the TCR and CD28 cell surface molecules (19). Allospecific T_s may, therefore, display their effector function by preventing the APC from upregulating the surface density of costimulatory molecules to the threshold required for inducing T_h proliferation. Such a mechanism is consistent with the finding that the capacity of T_h to display proliferative responses in the presence of T_s was restored upon ligation of CD28 by anti-CD28 mAb. Previous studies have shown that blocking the interaction of CD28 with CD80/CD86 either by use of anti-CD28 mAb Fab fragments or CTLA-4-Ig, leads to sustained T cell hyporesponsiveness to the specific alloantigen in MLC (20). Both agents blocked T cell alloreactivity and achieved a similar degree of inhibition on naive and memory T cells. However, T cell responsiveness was not completely abolished suggesting that other CD28- independent costimulatory pathways contributed to T_h alloactivation. Residual T_h proliferation in the presence of suppressor cells was also observed in our experiments, consistent with this possibility.

10

15

20

25

30

35

The fact that the CD8⁺ subset of lymphocytes contains a CD28⁺ population with alloantigen-specific cytotoxic activity and a CD28⁻ population with suppressor activity has been previously described (21). However, the suppressive effect of CD8⁺CD28⁻ cells as tested in primary MLC was found to be specific for HLA-DR antigens of the allogeneic target rather than for HLA class I antigens. Since CD8 molecule serves as

coreceptor for T cells recognizing MHC-class I molecules, it is difficult to understand how allospecific Ts carrying the CD8⁺CD28⁻ phenotype recognize and react against HLA-DR antigens.

5

The finding of the present study that allospecific CD8⁺ CD28⁻ Ts recognize on target APCs HLA class I antigens, and that they suppress the response of CD4⁺ Th to HLA-class II antigens of the same APC, inhibiting upregulation of CD80/CD86 expression, provides a reasonable explanation for the suppressor effect.

10
15
20
25
30
35

Several lines of evidence have demonstrated that molecular mimicry, an extensively discussed phenomenon (22), represents the main mechanism for direct allorecognition (23). Alloreactive T cell clones often display dual recognition ability for a nominal antigen and an alloantigen (24,25). This indicates that allogeneic MHC antigens can mimic immunogenic peptide/self MHC complexes which activate self-restricted T cells. It is, therefore, likely that allo-MHC class I restricted Ts regulate not only T helper/inducer reactivity against allogeneic HLA-DR antigens, but also Th reactivity against other antigenic peptides bound to self-MHC class II molecules. In this context the molecular characterization of MHC class I bound peptides which activate Ts may have important implications for the treatment of autoimmune and infectious diseases. While suppression of autoimmunity may be accomplished by priming CD8⁺CD28⁻ T cells with suppressor-inducing peptides, ablation of such a population may be required for treatment of certain infectious diseases. The observation that the proportion of CD8⁺CD28⁻ T cells within the CD8⁺ subset increases in HIV+ individuals as the disease progresses (26), supports the notion that this population of cells has important immunoregulatory function, and that it may depress Th reactivity.

Very recently it was shown that chronic activation of both

human and murine CD4⁺ T cells in the presence of IL-10 generates antigen specific T_s which produce high level of IL-10 and inhibit T cell responses to allogeneic monocytes. No direct interaction between T_n, T_s and APC was required in this system. These CD4⁺ T regulatory cells were shown to inhibit antigen-specific immune responses through the secretion of IL-10 and TGF- β (27). Hence, regulatory T cells operating by different mechanisms exist within the CD4 and CD8 subset.

10

The present study demonstrates for the first time that allospecific and xenospecific T_s can be generated from any individual by in vitro education and expansion. Preliminary experiments indicate that large numbers of T_s can be obtained in cultures. Since allospecific and xenospecific CD8⁺CD28⁻ T_s inhibit both the recognition and memory response of CD4⁺ T cells in primary and secondary MLCs, it is possible that generation of Ts can provide a tool for prevention and suppression of transplant rejection. "Adoptive transfer" of autologous CD8⁺ CD28⁻ T_s primed in vitro with donor APC may confer specific immunologic tolerance to human recipients of allogeneic or xenogeneic transplants.

15

20

References for First Series of Experiments

5 1. Nisco, S.J., Hissink, R.T., Vriens, P.W., Hoyt, E.G., Reitz, B.A., and Clayberger, C. (1995) In vivo studies of the maintenance of peripheral transplant tolerance after cyclosporine. *Transplantation* 59: 1444.

10 2. Field, E.H., Rouse, T.M., Gao, Q. and Chang, B. (1997) Association between enhanced Th2/Th1 cytokine profile and donor T-cell chimerism following total lymphoid irradiation. *Human Immunol.* 52: 144.

15 3. Strober, S. (1984) Natural suppressor (NS) cells, neonatal tolerance and total lymphoid irradiation: exploring obscure relationships. *Annu. Rev. Immunol.* 2, 219.

20 4. Maki, T., Simpson, M., and Monaco, A. P. (1982.) Development of suppressor T cells by anti-lymphocyte serum treatment in mice. *Transplantation* 34: 376.

25 5. Qin, S., Cobbold, S.P., Pope, H., Elliott, J., Kioussis, D., Davies, J., and Waldmann, H. (1993) 'Infectious' transplantation tolerance. *Science* 259: 974.

30 6. de Waal, L.P., and van Twuyver, E. (1991) Blood transfusion and allograft survival: Is mixed chimerism the solution for tolerance induction in clinical transplantation? *Immunol.* 10: 417.

35 7. Saitovitch, D., Bushell, A., Mabbs, D.W., Morris, P.J. and Wood, K.J. (1996) Kinetics of induction of transplantation tolerance with a nondepleting anti-CD4 monoclonal antibody and donor-specific transfusion before transplantation. *Transplantation* 61: 1642.

8. Shoskes, D.A. and Wood, K.J. (1994) Indirect presentation of MHC antigens in transplantation. *Immunol. Today* 15: 32.

9. Hall, B.M., Pearce, N.W., Gurley, K.E. and Dorsch, S.E. (1990) Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *J. Exp. Med.* 171: 141.

5

10 10. Roser, B.J. (1989) Cellular mechanisms in neonatal and adult tolerance. *Immunol. Rev.* 107: 179.

11. Padberg, W.M., Lord, R.H., Kupiec-Weglinski, J.W., Williams, J.M., Di Stefano, R., Thornburg, L.E., Araneda, D., Storm, T.B. and Tilney, N.L. (1987) Two phenotypically distinct populations of T cells have suppressor capabilities simultaneously in the maintenance phase of immunologic enhancement. *J. Immunol.* 138: 1751.

12. Mosmann, T. R., Cherwinski, H., Bond, M.W., Giedlin, M. A. and Coffman, R.L. (1986) Two types of murine helper T cell clone: I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136: 2348.

20

13. Lorber, M.I., Wall, K.A., Loken, M.R. and Fitch, F.W. (1984) Control of cloned alloreactive T lymphocyte proliferative responses: A possible role for cell-surface-bound alloantigen. *Transplantation* 38: 361.

25

14. Talanian, R.V., Yang, X., Turbor, J., Seth, P., Ghayur, T., Casiano, C.A., and Froelich, C.J. (1997) Granule-mediated killing: Pathways for Granzyme B-initiated apoptosis. *J. Exp. Med.* 186:1323.

30

15. Linsley, P.S., and Ledbetter, J.A. (1993) The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* 11: 191.

35

16. Sayegh, M.H., Akalin, E., Hancock, W.W., Russell, M.E., Carpenter, C.B., Linsley, P.S. and Turka, L.A. (1997) CD28

-57-

- B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. J. Exp. Med. 185: 393.

5 17. Lanier, L.L., O'Fallon, S., Somoza, C., Philips, J.H., Linsley, P.S., Okumura, K., Ito, D. and Azuma, M. (1995) CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J. Immunol. 154: 97.

10 18. Lombardi, G., Sidhu, S., Batchelor, R., Lechler, R. (1994) Anergic T cells as suppressor cells in vitro. Science 264: 1587.

15 19. van der Merwe, P.A., Bodian, D.L., Daenke, S., Linsley, P.S., and Davis S.J. (1997) CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. J. Exp. Med. 185: 393.

20 20. Tan, P., Anasetti, C., Hansen, J.A., Melrose, J., Brunvand, M., Bradshaw, J., Ledbetter, J.A. and Linsley, P.S. (1993) Induction of alloantigen-specific hypo-responsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. J. Exp. Med. 177: 165.

25 21. Damle, N.K., Mohagheghpour, N., Hansen, J.A., and Engleman, E.G. (1993) Alloantigen-specific cytotoxic and suppressor T lymphocytes are derived from phenotypically distinct precursors. J. Immunol. 131: 2296.

30 22. De Berardinis, P., Guardiola, J. and Manca, F. (1997) Epitope context and reshaping of activated T helper cell repertoire. Human Immunol. 54: 189.

35 23. Benichou, G. and Fedoseyeva, E.V. (1996) The contribution of Peptides to T cell allore cognition and allograft rejection. Intern. Rev. Immunol. 13: 231.

24. Lechler, R.I., Heaton, T., Barber, L., Bal, V.,

Batchelor, J.R. and Lombardi, G. (1992) Molecular mimicry by major histocompatibility complex molecules and peptides accounts for some alloresponses. Immunol. Lett. 34: 63.

5 25. Liu, Z., Sun, Y.K., Xi, Y.P., Harris, P. and Suciu-Foca, N. (1992) T cell recognition of self-human histocompatibility leukocyte antigens (HLA)-DR peptides in context of syngeneic HLA-DR molecules. J. Exp. Med. 175: 1663.

10 26. Lloyd, T.E., Yang, L., Tank, D.N., Bennett, T., Schober, W. and Lewis, D.E. (1997) Regulation of CD28 co-stimulation in human CD8+ T cells. J. Immunol. 158: 1551.

15 27. Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E. and Roncarolo, M.G. (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389: 737.

Second Series of Experiments

The shortage of organ donors is an ever increasing problem in clinical transplantation. Although the use of pig organs may offer a solution, there are still several immunological barriers that should be overcome before xenotransplantation can be envisioned. The first is the hyperacute rejection caused by the binding of naturally occurring antibodies and complement, present in primates, to pig endothelial cells. Recent progress in the generation of transgenic pigs expressing human complement-regulatory molecules on vascular endothelium may solve this critical problem (1-4). However, T helper cell recognition of xenogeneic MHC antigens via the direct and indirect pathways is likely to result in strong cellular immune responses that may be difficult to suppress using currently available strategies (4, 5). It is therefore apparent that the development of methods for specific suppression of xenograft rejection is an important objective for achieving successful xenotransplantation.

Although immunologic tolerance to allogeneic and xenogeneic tissues has been induced in a variety of experimental models (2, 6), attempts to ablate specifically the immune response to HLA-incompatible transplants in human patients have failed thus far. However, two recent reports have described strategies for in vitro education of regulatory T cells that suppress in a specific manner the direct recognition by CD4⁺ T cells of MHC class II antigens expressed on allogeneic APCs (7, 8). In one of these studies, regulatory T cells with suppressor activity were generated by stimulation of CD4⁺ T cells with allogeneic monocytes in the presence of IL-10. These regulatory T cells inhibited specifically the reactivity of CD4⁺ T helper cells through the secretion of IL-10 and TGF- β (7).

In the other study, suppressor T cells were generated by multiple stimulations of human peripheral blood lymphocytes

-60-

(PBL) with allogeneic APCs and shown to display the CD8⁺CD28⁻ phenotype (8). These CD8⁺CD28⁻ T cells recognized specifically HLA class I antigens expressed by the stimulatory APCs and suppressed the proliferative response of alloreactive CD4⁺ T cells against APCs used for priming. The suppressive effect was not mediated by lymphokines but instead required cell-to-cell interaction between CD4⁺ T helper (Th) cells, CD8⁺CD28⁻ T suppressor (Ts)⁺ cells, and allogeneic APCs expressing antigens against which the T cells were primed. In this system, Ts cells appeared to act by inhibiting costimulatory signals delivered by the allogeneic APCs, such as those provided by CD80/CD86 molecules (8).

This report demonstrates that xenospecific suppressor T cells can be also generated by multiple in vitro stimulations of human T cells with pig PBMCs. The CD8⁺CD28⁻ population from these T cell lines (TCL) recognizes specifically xenogeneic MHC class I antigens and suppresses the proliferative response of Th cells to MHC class II antigens expressed by the xenogeneic APCs. Xenospecific Ts cells interfere with the expression of CD154, the CD40 ligand, on xenoreactive Th cells, further supporting the concept that the suppressor effect results from inhibition of costimulatory interactions between Th cells and APCs.

The abbreviations used herein are as follows: PI=Propidium Iodide; TCL=T Cell Lines; Ts=T Suppressor cells; SLA = Swine Histocompatibility leukocyte antigen; and CD40L=CD40 Ligand.

Materials and Methods

Pig specimens. Blood was obtained from outbred pigs and from Yucatan miniature swine (Sinclair Research Center, Columbia, MO). MHC haplotypes were defined by RFLP using swine histocompatibility leukocyte antigen (SLA) class I- and class II-specific probes (9-11). For experiments aimed at the identification of MHC antigens recognized by xenospecific Ts

cells, blood was obtained from three SLA homozygous lines named W, Z, and Q. Line Q is homozygous for a crossover haplotype that carries the SLA class I genes of strain W and the SLA class II genes of Z (9-11).

5

Human specimens. Blood was obtained from healthy blood donors typed for HLA class I and class II antigens by conventional serology and by genomic typing of in vitro amplified DNA with sequence-specific oligonucleotide probes.

10

Generation of xenoreactive and alloreactive T cell lines. Human and pig PBMCS were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding human PBMCS (1×10^6 /ml) were stimulated in 24-well plates with irradiated (1600r) pig or human PBMCS (1×10^6 /ml). Cells were cocultured for 7 days in complete medium (RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2mM glutamine and 50 mg/ml gentamicin) (Gibco, Baltimore, MD). Responding cells were restimulated at seven day intervals in medium containing 10 U/ml rIL-2 (Boehringer Mannheim, Indianapolis, IN).

15

20

25

30

35

Cell separation. NK cells were depleted from the alloreactive or xenoreactive TCLs before testing using goat anti-mouse magnetic beads (Dynal, Lake Success, NY) coupled with mAb anti-CD16 and CD56 (Becton Dickinson, San Jose, CA). Suspensions used in blastogenesis assays contained < 2% CD16/CD56-positive cells, as indicated by flow cytometry. CD4⁺ and CD8⁺ T cells were separated from alloreactive and xenoreactive TCL by negative selection using Dynal CD4 and CD8 magnetic beads. T cell suspensions used as responders in blastogenesis assays were >98% positive for the CD4 and CD45RO markers. CD8⁺CD28⁻ T cell suspensions were prepared by depletion of CD28⁺ T cells from purified CD8⁺ T cell suspensions. For this procedure, goat anti-mouse Dynal beads were coupled with mAb anti-CD28 (Becton Dickinson, San Jose, CA), according to the manufacturer's instructions. The CD28-coupled beads were washed and incubated at 4×10^7

-62-

beads/ml with 1×10^7 CD8⁺ T cells for 20 minutes at 4°C, with gentle end-over-end mixing. Rosetted CD8⁺CD28⁺ T cells were detached from the beads by overnight incubation at 37°C and used in cell-mediated lysis experiments. Nonrosetted cells were collected, washed three times and resuspended at 2.5×10^5 cells/ml in complete RPMI 1640 culture medium. The purity of the suspension was monitored by cytofluorographic analysis. The suspension was reresetted with CD28 beads when necessary, to obtain a population contaminated by < 2% CD28⁺ bright cells.

Proliferation Assays. Blastogenesis assays were performed on day 14 or 21, after two or three stimulations, respectively, of human T cells with allogeneic or xenogeneic PBMCs. TCLs were then tested for reactivity to stimulating APCs either as nonfractionated, NK-depleted suspensions (5×10^4 cells/well) or as NK-depleted CD4⁺ T cell suspensions (2.5×10^4 cells/well). Responding cells were stimulated with irradiated allogeneic or xenogeneic PBMCs (5×10^4 cells/well). CD8⁺CD28⁻ T cells tested for suppressor activity were added to the cultures (1.25×10^4 cells/well) at the initiation of the blastogenesis assay. To study the dose-dependent effect of CD8⁺CD28⁻ T cells on Th cell proliferation, increasing concentrations of Ts cells were added to parallel cultures as indicated. Cultures were set-up in 96-well trays in a total volume of 0.2 ml. In some experiments, murine mAbs to human IL-10 (at 1 μ g/ml) or TGF- β (at 5 μ g/ml) from R&D Systems (Minneapolis, MN) were added to the cultures at the initiation of the assay. After 48 hours of incubation, the cultures were pulsed with [³H] thymidine ([³H]TdR) and harvested 18 hours later. [³H]TdR incorporation was determined by scintillation spectrometry in an LK Betaplate counter. Results were expressed as mean counts/min of triplicate reactions. Percent suppression was calculated as $1 - [(cpm \text{ in Th} + \text{Ts} + \text{APC cultures}) / (cpm \text{ in Th} + \text{APC cultures})]$.

Diffusion chamber experiments. Xenoreactive CD4⁺ T cells

(2.5×10^4 cells/well) and irradiated xenogeneic APCs (5×10^4 cells/well) were cocultured in the bottom compartment of a transwell system (Nalge Nunc International, Roskilde, Denmark). Xenospecific CD8 $^+$ CD28 $^-$ T cells (1.25×10^4 cells/well) were added either to the bottom compartment or cocultured with specific pig APCs in the top compartment of the transwell system. After 48 hours the semipermeable membranes were removed and the proliferative response of Th cells was measured by [3 H]TdR incorporation during the last 18 hours of culture.

Flow cytometry. Human T cell subsets were defined using mAb CD4, CD8, CD28, CD45RO, and CD16/56 (Becton Dickinson). Cell suspensions were phenotyped before testing with a FACScan flow cytometer instrument (Becton Dickinson) equipped with a 15-mm argon laser. CaliBRITE flow cytometer beads and FACSComp program (Becton Dickinson) were used for calibration of the cytometer.

To study the expression of CD154 on responding human Th cells, cells were incubated for 6 or 18 hours in MLC and then stained with saturating amounts of mAbs CD3-Per CP (peridinin chlorophyll protein-conjugated anti-CD3 mAb), CD154-PE and CD4-FITC or CD8-FITC (Becton Dickinson).

Cells were analyzed with CellQuest software on a 650 Apple Macintosh computer. Five parameter analysis (forward scatter, side scatter and three fluorescence channels) were used for list mode data analysis. The FL3 channel was used as fluorescence trigger, FL1 and FL2 were used as analysis parameters.

The cytokine profile of xenoreactive Th and Ts cells was determined by flow cytometry. CD4 $^+$ Th cells and CD8 $^+$ CD28 $^-$ Ts cells were isolated from TCLs and activated in 4 hour cultures with 25 ng/ml PMA and 1 μ g/ml of ionomycin. Brefeldin A (Sigma Chemical, St. Louis, MO) was added at 10 μ g/ml for the last 2 hours of incubation to inhibit

-64-

intracellular transport. Cells were fixed and stained for detection of intracellular cytokines using mAbs IL-2-FITC, IFN- γ FITC, IL-4 PE (Becton Dickinson), and IL-10 PE (R&D Systems).

5

Study of Apoptosis. The ability of xenoreactive CD8 $^{+}$ CD28 $^{-}$ human Ts cells to induce apoptosis of pig PBMCs and of xenoreactive human CD4 $^{+}$ Th cells after 4 hours of coincubation at 37°C was tested by flow cytometry with the use of annexin V as a marker for apoptotic cells. As positive controls, cells treated with camptothecin (Sigma) were used. The ratio of pig PBMC, human Th cells and human Ts cells was 1:0.5:0.25, as also used in blastogenesis assays. After incubation, cells were stained with mAb anti-human CD3- PE or CD4- PE, washed and subsequently stained with annexin V-FITC and propidium iodide (PI) (R&D Systems). To analyze the population of pig PBMCs, log FL2 (CD3- PE) versus side scatter parameters were used to gate out human CD3 $^{+}$ T cells. The percentage of apoptotic pig cells was determined from log FL1 (annexin-FITC) versus FL3 (PI) dot plots. To analyze the population of human CD4 $^{+}$ Th cells undergoing apoptosis, log FL2 (CD4-PE) versus side scatter parameters were used to gate on CD4 positive cells. Log FL1 (annexin V-FITC) versus FL3 (PI) dot plots of the gated population provided the percentage of apoptotic CD4 $^{+}$ Th cells.

30

Cytotoxicity Assays. CD8 $^{+}$ CD28 $^{-}$ and CD8 $^{+}$ CD28 $^{+}$ were isolated from activated CD8 $^{+}$ cells and tested for cytotoxicity in a ^{51}Cr release assay. Target cells were pig PBMCs stimulated with PHA (2 $\mu\text{g}/\text{ml}$) 3 days before the cytotoxicity assay. The cytotoxicity assay was performed with different effector to target cell ratios (E:T).

35

The percent cytotoxicity was calculated as % Lysis =

-65-

100 X { [Experimental release (cpm) - Spontaneous release (cpm)] / [Maximum release (cpm) - Spontaneous release (cpm)] }.

TCR Spectratyping

5 Total RNA was extracted using QIAGEN columns (QIAGEN Inc., Valencia, CA) from xenoreactive human CD8⁺CD28⁺ Ts cells. RNA was reverse transcribed into cDNA in a reaction using Moloney murine leukemia virus reverse transcriptase primed with oligo(dT)₁₂ (Clontech Laboratories Inc., Palo Alto, CA), as recommended by the manufacturer.

10 Aliquots of the cDNA synthesis reaction were amplified in 50-ml reactions with each of the 24 V β oligonucleotides (0.5 μ M final concentration) and the C β oligonucleotide (0.5 μ M final concentration). V β and C β primers were previously described (12, 13). As an internal control for the amount of cDNA used per reaction, a tube containing sense and antisense primers for the first exon of C β region was included. Two microliters of the V β -C β PCR products were subjected to elongation with a fluorophore-labeled C β or J β -specific primer (0.5 μ M final concentration) (12). The size and fluorescence intensity of labeled runoff products were determined on a 377 DNA sequencer (Perkin Elmer Applied Biosystem Division, Foster City, CA) and analyzed by ABI PRISM 377 GENESCAN Analysis Program (Perkin Elmer Applied Biosystem Division) (13).

15 The relative intensity of each V β family or J β -V β fragment was calculated as the peak area corresponding to each V β family or J β -V β fragment divided by the sum of all area peaks (12).

Statistical Analysis

20 Statistical analysis of the results was performed using BMDP statistical software. Analysis of variance to assess significance of group differences (ANOVA) followed by Tukey's method for multiple comparison was applied. Correlation coefficients were obtained using Linear Regression Analysis.

-66-

Student's *t* test of significance was also used to access the differences between groups.

RESULTS

Specificity of Xenoreactive T Suppressor (Ts) Cells

TCLs were generated by priming T cells from a healthy volunteer (SA) with PBMCS from an unrelated blood donor (BM) or with PBMCS from an outbred pig (pig A). The allospecific TCL (SA-anti-BM) as well as the xenospecific TCL (SA-anti-pig A) showed higher reactivity against APCs from the original stimulator after removal of CD8⁺CD28⁻ Ts cells from the suspensions (Fig. 6). Furthermore, when CD8⁺CD28⁻ T cells were added to the cultures at the initiation of the blastogenesis assay, they inhibited significantly ($p<0.05$) the reactivity of CD4⁺ Th cells against APCs used for priming. The suppressive effect was species-specific since CD8⁺CD28⁻ Ts cells primed to pig APCs did not inhibit the response of CD4⁺ Th cells primed to human APCs. Similarly, Ts cells primed to human APCs did not inhibit the response of CD4 Th cells primed to pig APCs, indicating that Ts cells recognize species-specific antigens (Fig. 6). Studies of an additional four xenospecific and allospecific TCLs yielded similar results.

To determine whether the suppressive effect correlates with the number of Ts cells present in the cultures, Ts cells from two xenoreactive TCLs, MN-anti-pig B and AP-anti-pig B, were tested at various concentrations for their ability to inhibit proliferation of Th cells from TCLs MN-anti-pig B and AP-anti-pig B, respectively. As illustrated in Table 5, the strength of the suppressive effect increased with the number of Ts cells, indicating that suppression was dose dependent ($r = 0.85$, $p < 0.008$).

To determine the nature of the SLA antigens recognized by CD8⁺CD28⁻ T cells on pig stimulating cells, xenoreactive TCLs

-67-

were generated by stimulating PBMCs from a human blood donor (ES) with irradiated APCs from three different strains of inbred swine: Q, W, and Z. Strain Q shares class I antigens with W and class II antigens with Z, being homozygous for a recombinant haplotype which carries the SLA class I antigens of W and the class II antigens of Z.

Table 6 shows the results of independent experiments in which TCL generated on three different occasions, by priming PBMC from individual ES with APCs from strain Q, W, and Z, were used.

-68-

Table 5.Dose-Dependent Suppression of CD4⁺ Th Reactivity to Irradiated Pig APCs in the Presence of CD8⁺CD28⁻ Ts.

5

	Number of cells/well			Reactivity (mean cpm) of Th cells from TCL*	
	<u>APCs</u>	<u>Th</u>	<u>Ts</u>	<u>MN-anti-pig B</u>	<u>AP-anti-pig B</u>
10	5×10^4	0	0	278	118
15	5×10^4	2.5×10^4	0	27,031	13,306
	5×10^4	2.5×10^4	1.25×10^4	13,808	6,394
	5×10^4	2.5×10^4	2.5×10^4	8,516	2,848
20	5×10^4	2.5×10^4	5.0×10^4	6,480	2,689

25

* All reactions were performed in triplicate. The SD to the mean was <10%.

Table 6. Reactivity of CD4+ Th Cells from TCL ES-anti-swine Q in the Presence of CD8+CD28- T Suppressor Cells

Ts from	Source of APCs used for priming of Ts	Genotype of APCs		Q	Reactivity (mean cpm) of CD4+ Th from TCL ES-anti-Q*		
		Class I	Class II		W	Z	W+Z
<u>Experiment 1</u>							
No Ts added	-	-	-	-	31,079	3,719	28,269
ES-anti-Q	Q	W	Z	7,301	1,746	-	38,040
ES-anti-W	W	W	W	12,140	2,711	25,545	32,280
ES-anti-Z	Z	Z	Z	25,946	2,709	17,018	-
<u>Experiment 2</u>							
No Ts added	-	-	-	-	33,787	-	32,880
ES-anti-Q	Q	W	Z	8,417	-	30,510	-
ES-anti-W	W	W	W	15,240	-	33,150	-
ES-anti-Z	Z	Z	Z	32,790	-	15,208	-
<u>Experiment 3</u>							
No Ts added	-	-	-	-	22,540	1,452	20,715
ES-anti-Q	Q	W	Z	5,215	1,310	25,487	19,830
EA-anti-W	W	W	W	7,523	1,640	22,519	20,156
ES-anti-Z	Z	Z	Z	19,834	1,415	8,328	9,780
							9,530

* For each experiment, reactions were set up in triplicate cultures. The SD to the mean of triplicate reactions was <10%.

-70-

In these experiments CD4⁺ Th cells from ES-anti-swine Q were tested for reactivity in cultures without Ts cells or with Ts cells from ES-anti-Q, ES-anti-W and ES-anti-Z.

5 The reactivity of Th cells primed to APCs of strain Q to the specific stimulator Q was inhibited efficiently by autologous Ts cells primed to Q or to W (which shares MHC class I antigens with Q), but not by Ts cells primed to Z (which is MHC class II identical, yet class I different from the specific stimulator Q) ($p<0.05$). CD4⁺ T cell reactivity to strain Z was inhibited only by Ts cells primed to Z, but not by Ts cells primed to strain Q or W which are class I different from Z ($p<0.05$) (Table 6). This indicates that
10 CD8⁺CD28⁺ Ts cells are activated by SLA class I antigens on xenogeneic APCs and inhibit the response of CD4⁺ Th cells against class II antigens expressed by the same stimulating target cells. The MHC class II specificity of Th cell reactivity was confirmed by the fact that human CD4⁺ T cells primed to APCs from a strain Q swine reacted to APCs from strain Z (class II identical with Q) but not from strain W (class II different from Q).

15

20

25 To establish whether the suppressive activity of CD8⁺CD28⁺ T cells requires the direct interaction of these cells with the APCs that trigger Th cells reactivity, cell-mixing experiments were performed. In these experiments, mixtures of APCs from strain Z and W were used to stimulate the reactivity of Th cells from TCL ES-anti-Q. The reactivity of Th cells anti-Q was tested in cultures with or without Ts cells primed to Q, W, or Z. In cultures without Ts cells, Th cells primed to Q proliferated vigorously, consistent with the specific recognition of MHC class II antigens shared by strains Q and Z. This response, however, was not inhibited by Ts cells primed to Q or W, indicating that Ts cells do not inhibit Th cell reactivity to SLA class II antigens unless the SLA class I antigens which they recognize are coexpressed by the same APCs. Indeed inhibition of the response to mixtures of APCs from W and Z was observed only
30

35

- 71 -

in the presence of Ts cells primed to Z ($p<0.05$), further demonstrating that the interaction of Ts cells and Th cells with the same APCs is required for suppression. This finding is consistent with the hypothesis that Ts cells interfere with the delivery of costimulatory signals by APCs to CD4 $^{+}$ Th cells(8).

It is possible, however, that in addition to interacting with APCs, Ts cells and Th cells also "communicate" with each other, recognizing TCR determinants or other structures in an MHC-restricted manner (14, 15). To explore this possibility, TCLs were generated by stimulating PBMCS from two HLA-disparate individuals, AP (HLA-A30, B35, DRb1*0701, 1301) and MN (HLA-A1, A32, B8, B44, DRb1*0101, 0301) with APCs from the same outbred pig (pig B). The blastogenic response of both TCLs (MN-anti-pig B and AP-anti-pig B) to pig APCs was significantly stronger ($p<0.01$) when CD8 $^{+}$ CD28 $^{-}$ Ts cells were depleted from the cell suspensions, indicating that CD4 $^{+}$ Th cell responses were suppressed by autologous Ts cells (Fig. 7). The reactivity of CD4 $^{+}$ Th cells from both lines to stimulating APCs was inhibited by CD8 $^{+}$ CD28 $^{-}$ Ts cells from either of these lines ($p<0.01$). The difference between the suppressor activity of Ts cells from MN-anti-pig B and AP-anti-pig B was not statistically significant. These results were confirmed in two additional experiments for which other TCL were used. Hence, no MHC-restricted interaction between Th cells and Ts cells is required for suppression to occur.

To determine whether Ts cells secrete inhibitory factors, coculture experiments using semipermeable membranes, for separating Ts cells from Th cells, were performed. Th cells from TCL ES-anti-W were stimulated with irradiated xenogeneic APC from strain W in the bottom compartment, whereas Ts cells were stimulated with the same APCs in the top compartment. Xenoantigen-specific stimulation of Th cells was inhibited significantly only when Ts cells, Th cells, and APCs were in

-72-

close contact, but not when Ts cells and Th cells were separated by a membrane ($p=0.0001$), indicating that cell-to-cell interaction is required for the suppressive effect induced by CD8 $^{+}$ 28 $^{-}$ Ts cells to occur (Fig. 8A).

5

To further explore the possibility that suppression is mediated by inhibitory cytokines, such as IL-10 or TGF- β , experiments were performed in which mAbs to IL-10 and to TGF- β were added to cultures containing only Th cells, or both Th cells, Ts cells, and stimulating APCs. These mAbs had no significant effect on Th cells proliferation in the absence of Ts cells (data not shown) and failed to abrogate or decrease the inhibitory effect induced by Ts cells on Th cell reactivity (Fig. 8B).

10

15 Cytofluorographic analysis of Ts cells from three different TCLs (ES-anti-Q, ES-anti-W and ES-anti-Z) showed that they produced high levels of IFN- γ and moderate amounts of IL-2, yet no detectable levels of IL-4 and IL-10. Th cells from the same cultures produced high levels of IL-2 and IFN- γ , moderate amounts of IL-4, and no IL-10 (Fig. 9).

20

Study of Ts Cell-Induced Apoptosis

25

The possibility that the suppressive activity of xenospecific Ts may be due to killing of pig APCs was explored. Ts cells from a human TCL (GC-anti-swine Z), which inhibited by 88% the response of autologous Th cells to the specific stimulator, were tested for their ability to induce apoptosis or lysis of pig APC. Flow cytometry studies of apoptosis were performed by incubating Ts cells for 4 hours with pig APCs in the presence or absence of xenoreactive CD4 $^{+}$ Th cells and then staining the cultures with annexin V. The percentage of annexin V positive APCs was not significantly different in cultures with or without Ts cells, indicating that no apoptosis of pig APCs was induced (Fig. 10A). Also, the percentage of necrotic pig cells stained by PI was not significantly different in cultures with or without human Ts

30

35

- 73 -

cells. Furthermore, cell mediated lysis experiments in which PHA-activated pig lymphocytes were used as targets showed lysis when CD8⁺CD28⁺ T cells were used as effectors, but not when CD8⁺CD28⁻ T cells from the same line were tested. This
5 demonstrates that Ts cells do not kill xenogeneic APCs used for priming (Fig. 10B).

Next investigated was the hypothesis that Ts cells may cause apoptotic death of xenoreactive Th cells. In these
10 experiments, CD4⁺ Th cells from TCL GC-anti-swine Z were incubated for 4 hours with APCs from swine Z, in the presence or absence of autologous Ts cells. The percentage of annexin V-positive CD4⁺ Th cells in cultures with Ts cells was not significantly different from the percentage found in cultures without Ts cells, indicating that suppression is not mediated by killing of xenoreactive Th cells (Fig. 10C). Hence, Ts cell suppressive activity is not due to killing of either Th cells or stimulatory APCs.
15

20 Expression of CD40 Ligand (CD40L) (CD154) on Xenoreactive Th Cells

The possibility that Ts cells interfere with the costimulatory interaction between CD154 on Th cells (CD40L, T-BAM, p39, or TRAP) and CD40 on xenogeneic APCs has been explored. For this, the expression of CD154 on xenoreactive CD4⁺ Th cells which were stimulated with pig APCs in the presence or in the absence of Ts cells was studied. After 6 hours of incubation, cells were stained with mAbs anti-CD3, CD154, and either CD4 or CD8. Analysis of the results obtained in independent experiments, using six different TCLs, showed that the level of CD154 expression on CD4⁺ Th cells was significantly higher ($p<0.01$) in cultures containing pig APCs than in cultures without stimulating cells (Fig. 11A and 11B). However, expression of CD154 on CD4⁺ Th cells was drastically reduced in the presence of Ts cells (Fig. 11C), indicating that Ts cells prevent antigen-induced up-regulation of CD154 on CD4⁺ Th cells.
25
30
35

- 74 -

There was a statistically significant difference between the level of CD154 expression on Th cell cultures with and without Ts cells ($p<0.01$) in all six experiments. The up-regulation of CD154 was antigen specific, requiring TCR activation, since it did not occur on CD4⁺ Th cells challenged with APCs from an SLA class II-different pig (Fig. 11D). The expression of CD154 on xenoreactive CD4⁺ Th cells was maximal after 6 hours and decreased significantly after 18 hours of incubation with stimulating APCs (data not shown). No expression of CD154 was observed on Ts cells at any time point studied. Hence, Ts cell-induced events that result in Th cell inhibition occur within the first 6 hours of stimulation.

15 Spectratyping of TCLs Expressed by Ts

The V β gene usage of Ts cells from four human anti-pig TCLs (MN-anti-pig B, ES-anti-Q, ES-anti-W, and ES-anti-Z) was determined by spectratyping (Figs. 12 and 13). Ts cells from each of these xenoreactive TCL showed a restricted TCR V β gene usage. The side-by-side comparison of the V β repertoire expressed in unstimulated and stimulated CD8⁺CD28⁺ T cells indicates that after two stimulations with xenogeneic APCs, there was oligoclonal expansion of Ts cells, as illustrated in Fig. 12 and 13.

30 The V β 9 and V β 23 families were expressed by all TCLs yet with different relative intensities (Fig. 12 and 13). The relative intensities of V β 9 family in TCL MN-anti-pig B, ES-anti-Q, ES-anti-W, ES-anti-Z were 0.22, 0.19, 0.05, and 0.07, respectively, and the relative intensity of V β 23 was 0.48, 0.38, 0.30, 0.23, respectively. V β 16 was highly represented in all TCL generated from individual ES yet was absent in TCL obtained from another human subject (MN). V β 5 was also expressed by Ts cells from all TCL derived from responder ES, but with lower intensity than V β 16. In two of the TCLs from individual ES (ES-anti-pig W and ES-anti-pig Z), V β 14 was represented with high intensity (Fig. 13, B and

- 75 -

C). Other V β families, such as V β 15 were uniquely represented in one suppressor cell line (ES-anti-pig Z) (Fig. 13C), while absent from the other lines (ES-anti-Q, ES-anti-W and MN-anti-pig B). Analysis of the CDR3 size distribution revealed a unimodal or bimodal distribution for each V β family, except V β 23 that showed a multipeak gaussian-like distribution (Fig. 14). The oligoclonality of the V β repertoire expressed by xenoreactive Ts cells was also confirmed by analyzing the J β -V β fragments of V β families (Fig. 15).

10

DISCUSSION

15

The phenotypic characteristics of suppressor cells as well as the mechanisms that underlie their function have been the object of numerous studies (16-25). Both CD4 $^{+}$ and CD8 $^{+}$ T cells with suppressive activity have been described, although antigen-specific suppressor cell lines have been difficult to generate.

20

25

30

35

CD4 $^{+}$ T cells producing TGF- β , IL-4, and IL-10 were shown to play an important role in protecting animals from experimental autoimmune encephalomyelitis (EAE) after oral feeding with antigen (21). IL-10 was recently shown to induce *in vitro* differentiation of regulatory CD4 $^{+}$ T cells with suppressor activity and inhibit alloantigen-specific reactivity of CD8 $^{+}$ T cells (7, 23). MHC class-II restricted CD8 $^{+}$ Ts cells that release IL-4 and suppress Th1 cell proliferation were described in human leprosy (24, 25). In the mouse model, CD8 $^{+}$ Ts cells were also described, yet these cells were restricted by nonclassical MHC class I antigens (Qa-1) expressed by B cells and inhibited Th2 responses by production of IFN- γ (18). In other studies, suppression was mediated by Qa-1 restricted CD8 $^{+}$ T cells, which recognize TCR determinants on the membrane of CD4 $^{+}$ Th cells (14, 15, 25). The mechanism of antiidiotypic suppression involved Th cell lysis or induction of Th cell apoptosis via ligation of Fas (15, 19).

An alternative mechanism of suppression seems to reside in inhibition of TCR-mediated cytotoxicity by CD8⁺CD28⁻ and CD4⁺CD28⁻ T cells which express NK inhibitory receptors 5 (26-28). The inhibitory effect of these killing-inhibitory receptors results from mobilization of protein tyrosine phosphatases on the cytoplasmic tail of killing-inhibitory receptor molecules (28).

10 In a previous study it was shown that human CD8⁺CD28⁻ Ts cells, which inhibit alloreactive CD4⁺ Th cells, recognize HLA class I antigens on the surface of allogeneic APCs used for priming (8). The suppression was mediated by downregulation of CD80 and CD86 expression on the allogeneic APCs and, thus, by impairment of their ability to deliver the costimulatory signals required for the activation of CD4⁺ Th cells in response to HLA class II alloantigens.

15
20 The present study demonstrates for the first time that the xenospecific response of human CD4⁺ Th cells to pig MHC class II antigens can be also suppressed by CD8⁺CD28⁻ T cells immunized in vitro against xenogeneic MHC class I antigens. The suppressive effect was not mediated by idiotypic interactions between xenoreactive Ts cells and Th cells, 25 since Th cells primed to APCs from an individual pig were efficiently suppressed not only by autologous but also by allogeneic human Ts cells immunized against the same SLA class I antigens.

30 The possibility that suppression of CD4⁺ Th cells was mediated by lymphokines secreted by CD8⁺CD28⁻ Ts cells is also unlikely, since the suppressive activity required the interaction between Th cells and Ts cells with the same APCs. Thus, Th cell inhibition occurred only when the immunizing 35 SLA class I and class II antigens were coexpressed on the membrane of stimulating APCs, but not when these antigens were expressed by two distinct populations of APCs. Furthermore, diffusion chamber experiments in which Ts and

-77-

Th cells were separated by semipermeable membranes showed that Th cell reactivity to xenogeneic APCs was not inhibited, indicating that suppression is not mediated by soluble factors.

5

Cytofluorographic analysis of CD8⁺CD28⁻ Ts cells showed that these cells produce IL-2 and IFN- γ , but not IL-4 and IL-10. Moreover, experiments using mAbs against inhibitory cytokines, such as IL-10 and TGF- β , excluded their contribution to the suppressor effect. Hence, neither the production or consumption of lymphokines by Ts cells (22, 24, 29) can explain their inhibitory effect on Th cells in this system.

10

In the allogeneic system, it was demonstrated that Ts cells interfere with Th cell-induced up-regulation of B7 (CD80, CD86) expression on APC (8). The interaction between CD40 on APC and CD40L (CD154), a transiently expressed CD4⁺ T cell molecule, is essential for the induction of accessory molecules on APCs, in particular CD80, CD86, and 4-1BB ligand, and for the initiation of antigen-specific T cell reactivity (30-35). However, blockade of either CD28/B7 or CD40L/CD40 pathways does not inhibit completely T cell mediated alloimmune responses, indicating that, although interrelated, the CD28 and CD40L pathways serve as independent regulators of T cell responses (36).

20

25

30

35

The possibility that Ts cells interfere with the expression of CD40L (CD154) on activated CD4⁺ Th cells has been explored. Cytofluorographic analysis showed that up-regulation of CD154 expression on xenoreactive CD4⁺Th cells was induced by pig APCs, indicating that human CD154/pig CD40 interaction contributes to the strong proliferative response occurring on recognition by human TCRs of SLA class II antigens. Hence, in the human-pig system, xenoantigen-specific CD4⁺ Th cell responses involve not only the CD28/B7 and CD2/LFA1 costimulatory pathways, as previously described (37), but also the CD154/CD40 pathway.

-78-

However, the expression of CD154 on xenoreactive CD4⁺ Th cells was significantly reduced in the presence of Ts cells. The molecular mechanism of CD154 down-regulation on xenoreactive CD4⁺ Th cells by Ts cells is currently under investigation. The possibility that Ts cells prevent up-regulation of CD40L on CD4+ T cells by killing the xenogeneic stimulating cells or by inducing Th cell apoptosis was ruled out since no evidence of Ts cell-induced cell death was found by either flow cytometry or ⁵¹Cr release studies.

Proliferation of CD4⁺ T cells was not restored in the presence of cells expressing constitutively CD40L, suggesting that costimulation of xenogeneic APCs through the CD40-CD40L pathway is not sufficient to circumvent the suppressive effect of Ts cells (A.I. Colovai, manuscript in preparation). Down-modulation of CD154 by Ts cells may lead, however, to disengagement of Th cells from the targets, preventing full activation and proliferation of these cells.

T cell reactivity to allogeneic and xenogeneic MHC antigens bears resemblance to TCR activation by nominal antigens and pathogens, as it involves recognition of targets expressing novel MHC/peptide complexes. Since the generation of allo- or xenospecific Ts cells in vitro requires multiple rounds of stimulation, it is possible that the chronic exposure to antigen is also required in vivo for the induction of Ts cells. The oligoclonal expansion of a few TCR V β families observed within the population of xenoreactive Ts cells is reminiscent of the skewed TCR repertoire displayed by T lymphocytes with HLA class I-specific NK-inhibitory receptors (27), a phenomenon suggested to result from chronic antigenic stimulation.

MHC class I-restricted Ts cells may play a physiologic role in regulating the immune response of Th cells against self or non-self peptide/MHC class II complexes. The finding that there is cross-talk between the MHC class I and class II pathways of peptide processing supports the notion that the same APCs present both helper- and suppressor-inducing

- 79 -

peptides (38). It is possible that recognition by Ts cells of MHC class I-bound peptides helps control local inflammation caused by antigen-specific Th cells. Identification of suppressor-inducing peptides may be useful 5 for induction of unresponsiveness to auto-, allo-, or xenoantigens. Furthermore, understanding of the mechanism of Ts cell-mediated down-regulation of CD154 expression on activated Th cells may contribute to the development of new immunotherapeutic strategies.

10

15

20

This issue becomes particularly important in view of the recent finding that Th cells condition the APCs to directly stimulate T killer cells by CD154-CD40 signaling, rather than by delivering short range acting lymphokines (39-42). The emerging picture from our studies is that Ts cells down-regulate the immune response by interfering with CD154-CD40 signaling, thus preventing the up-regulation of costimulatory (B7) molecules on APCs.

-80-

References for the Second Series of Experiments

1. Sachs, D. H. 1994. The pig as a xenograft donor. *Path Biol.* 42: 217.
5
2. Cozzi, E. and D. J. G. White. 1995. The generation of transgenic pigs as potential organ donors for humans. *Nature Medicine.* 1:964.
- 10 3. Morgan, B. P. 1995. Complement regulatory molecules: application to therapy and transplantation. *Immunol. Today.* 16:257.
- 15 4. Platt, J. L. 1996. The immunological barriers to xenotransplantation. *Critical Rev. Immunol.* 16:331.
- 20 5. Choo, J. K., J. D. Seebach, V. Nickeleit, A. Shimizu, H. Lei, D. H. Sachs, and J. C. Madsen. 1997. Species differences in the expression of major histocompatibility complex class II antigens on coronary artery endothelium. *Transplantation.* 64:1315.
- 25 6. Cobbold, S. P., E. Adams, S. E. Marshall, J. D. Davies, and H. Waldmann. 1996. Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8. *Immunol. Rev.* 149:6.
- 30 7. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature.* 389:737.
8. Liu, Z., S. Tugulea, R. Cortesini, and N. Suciu-Foca. 1998. Specific suppression of T helper alloreactivity by

-81-

allo-MHC-class I restricted CD8⁺CD28⁺ T cells. *Int. Immunol.* 10:101.

5 9. Singer, D. S., R. Ehrlich, L. Satz, W. Frels, J. Bluestone, R. Hodes, and S. Rudikoff. 1987. Structure and expression of class I MHC genes in the miniature swine. *Vet. Immun. and Immunopath.* 17:211.

10 10. Hirsh, F., S. Germana, K. Gustafsson, K. Pratt, D. H. Sachs, and C. Laguern. 1992. Structure and expression of class II alpha genes in miniature swine. *J. Immunol.* 149:841.

15 11. Gustafsson, K., S. Germana, F. Hirsch, K. Platt, C. Laguern, and D. H. Sachs. 1990. Structure of miniature swine class II DRB genes: conservation of hypervariable amino acid residues between distantly related mammalian species. *Proc. Natl. Acad. Sci.* 87:9798.

20 12. Puiseux, I., J. Even, F. Joterau, M. Favrot, and P. Kourilsky. 1994. Oligoclonality of tumor infiltrating lymphocytes from human melanomas. *J. Immunol.* 153:2807.

25 13. Gardenet, L., D. Nicolas, C. Dovay, N. Chalumeau, V. Shaeffer, M. T. Zilber, A. Lim, J. Even, N. Mooney, C. Gelin, E. Gluckman, D. Charron, and A. Toubert. 1998. The umbilical blood ab T-cell repertoire: characteristics of a polyclonal and naive but completely formed repertoire. *Blood* 91:340.

30 14. Ware, R., H. Jiang, N. Braunstein, J. Kent, E. Wiener, B. Pernis, and L. Chess. 1995. Human CD8⁺ T lymphocyte clones specific for T cell receptor V_b families expressed on autologous CD4⁺ T cells. *Immunity.* 2:177.

35 15. Jiang, H., R. Ware, A. Stall, L. Flaherty, L. Chess,

-82-

and B. Pernis. 1995. Murine CD8⁺ T cells that specifically delete autologous CD4⁺ T cells expressing V β 8 TCR: a role of the Qa-1 molecule. *Immunity.* 2:185.

5 16. Jenkins, M. K., C. A. Chen, G. Jung, D. L Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol.* 144:16.

10 17. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science.* 248:1349.

15 18. Noble, A., Z. S. Zhao, and H. Cantor. 1998. Suppression of immune responses by CD8 cells. II. Qa-1 on activated B cells stimulate CD8 cell suppression of T helper 2 responses. *J. Immunol.* 160:566.

20 19. Noble, A., G. A. Pestano, and H. Cantor. 1998. Suppression of immune responses by CD8 cells. I. Superantigen-activated CD8 cells induce unidirectional Fas-mediated apoptosis of antigen-activated CD4 cells. *J. Immunol.* 159:559.

25 20. Barker, T. D., D. Weissman, J. A. Daucher, K. M. Roche, and A. S. Fauci. 1996. Identification of multiple and distinct CD8⁺ T cell suppressor activities: dichotomy between infected and uninfected individuals, evolution with progression of disease, and sensitivity to gamma irradiation. *J. Immunol.* 156:4476

30 35 21. Chen, Y., V. K. Kuchroo, J. I. Inobe, D. A. Hafler, H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science.* 265:1237.

-83-

22. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsetsofhuman CD4 and CD8 T cell clones. *Science* 254:279.

5

23. Groux, H., M. B., Bigler, J. E. de Vries, and M-G Roncarolo. 1998. Inhibitory and stimulatory effects of IL-10 on human CD8⁺ T cells. *J. Immunol.* 160:3188.

10

24. Bloom, B. R., R. I. Modlin, P. Salgame. 1992. Stigma variations: observations on suppressor T cells and leprosy. *Annu. Rev. Immunol.* 10:453.

15

25. Kuchroo, V. K., M. C. Byrne, Y. Astumi, E. Greenfield, J. B. Connolly, M. J. Whitters, R. M. O'Hara, Jr. M. Collins, and M. E. Dorf. 1991. T-cell receptor alpha chain plays a critical role in antigen-specific suppressor cell function. *Proc. Natl. Sci.* 88:8700.

20

26. Philips, J. H., J. E. Gumperz, P. Parham, and L. L. Lanier. 1995. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors of T lymphocytes. *Science*. 268:403.

25

27. Moretta, A., R. Biassoni, C. Bottino, D. Pende, M. Vitale, A. Poggi, M. C. Mingari, and L. Moretta. 1997. Major histocompatibility complex class-I specific receptors on human natural killer and T lymphocytes. *Immunol. Rev.* 155:105.

30

28. Strominger, J. L. 1997. Human NK cells: their ligand receptors and functions. *Immunol. Rev.* 155:119.

29. Lombardi, G., S. Sidhu, R. Batchelor, and R. Lechler. 1994. Anergic T cells as suppressor cells in vitro. *Science*. 264:1587.

-84-

30. Renheim, E. A., T. J. Kipps. 1993. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40 dependent signal. *J. Exp. Med.* 177:925.

5 31. Yang, Y., J. M. Wilson. 1996. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science*. 273:1862.

10 32. Lederman, S., M. J. Yellin, A. Krichevsky, J. Belko, J. J. Lee, and L. Chess. 1992. Identification of a novel surface protein on activated CD4⁺ T cells that induces contact dependent B cell differentiation (help). *J. Exp. Med.* 175:1091.

15 33. Grewal, I. S., J. Xu, and R. A. Flavell. 1995. Impairment of antigen-specific T cell priming in mice lacking CD40 ligand. *Nature*. 378: 617.

20 34. Jaiswal, A. I., and M. Croft. 1997. CD40 ligand induction on T cell subsets by peptide-presenting B cells. *J. Immunol.* 159: 2282.

25 35. DeBendette, M. A., A. Shahinian, T. W. Mak, and T. H. Watts. 1997. Costimulation of CD28⁻ T lymphocytes by 4-1BB ligand. *J. Immunol.* 158:551.

30 36. Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Rotchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P.S. Linsley, K. J. Winn, and T. C. Pearson. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381:434.

35 37. Murray, A. G., M. M. Khodadoust, J. S. Pober, and A. L. M. Bothwell. 1994. Porcine aortic endothelial cells activate human T cells: direct presentation of MHC antigens and costimulation by ligands from human CD2 and CD28.

-85-

Immunity. 1:57.

38. Harris, P. E., A. I. Colovai, A. Maffei, Z. Lui, N. Suciu-Foca. 1995. MHC class I presentation of exogenous and endogenous protein-derived peptides by a transfected human monocyte cell line. *Immunol.* 86:606.

5

39. Lanzavecchia, A. 1998. Licence to kill. *Nature* 393:413.

10

40. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature* 393:474.

15

41. Bennett, S. R. M., F. R. Carbone, F. Karamalis, R. A. Flavells, J. F. A. P. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signaling. *Nature* 393:478.

20

42. Schoenberger, S. P., R. E. M. Toes, E. I. H. van der Voort, R. Offringa, and C. J. M. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480.

25

43. J.K. Lunney and J.E. Butler, Immunogenetics, In The Genetics of the Pig, 1998, eds., M.F. Rothschild and A. Ruvinsky, CAB International.

-86-

Third Series of Experiments**INDUCTION OF MHC-CLASS I RESTRICTED HUMAN SUPPRESSOR T CELLS
BY PEPTIDE PRIMING IN VITRO**

5 A major goal in the treatment of T-cell mediated autoimmune diseases and allograft rejection is the development of antigen-specific immunosuppression. Induction of antigen-specific T suppressor cells may offer a means of preventing or treating pathogenic responses to self and allogeneic antigens without the complications associated with general immunosuppression.

10 Although suppressor T cell lines (TCL) are difficult to generate, the existence within the CD4⁺ and CD8⁺ T cell population of a functional subset of T lymphocytes that act to downregulate the immune response is well documented (1-5). Different mechanisms have been implicated, yet the cellular and molecular basis of antigen-specific suppression is still unclear (1-5). Recently, it has been demonstrated that 15 allospecific as well as xenospecific Ts can be generated by in vitro priming of human T cells with APCs from an individual of the same (allogeneic) or different (xenogeneic) species (7). Allospecific and xenospecific(6) Ts derive from the CD8⁺CD28⁻ subset and recognize specifically the 20 MHC-class I antigens expressed by the APCs used for priming (7).

25 Allospecific Ts prevent the upregulation of B7 molecules on target APCs, interfering with the CD28-B7 interaction required for T helper (Th) activation. Furthermore, these Th show no upregulation of the CD154 (CD40L) molecule when stimulated with the priming APCs in the presence of Ts, indicating that Ts also inhibit the CD154/CD40 costimulatory pathway (6) .

30 Cell mixture experiments showed that suppression of Th alloreactivity (7) and Th xenoreactivity (6) occurred only when the stimulatory APCs co-expressed MHC-class II antigens,

-87-

recognized by the Th to be suppressed, and MHC-class I antigens, recognized by the suppressor population. Hence the interaction of the APC with both Ts and Th is a necessary requirement for the development of the suppressor effect.

5

However, T cell reactivity against both allogeneic and xenogeneic MHC antigens in MLC, occurs primarily via the direct recognition pathway, which is not restricted by the responder's self MHC antigens. Ts-induced alterations of the APCs used for priming may, therefore, represent a peculiarity of the direct recognition pathway.

10

15

The aim of the present study was to determine whether MHC-class I restricted T cells with suppressor function can be also induced by T cell priming *in vitro* with nominal antigens such as Tetanus Toxoid and synthetic peptides.

20

Reported in the third series of experiments is the *in vitro* generation and characterization of self MHC restricted Ts which recognize antigens that have been processed and presented by autologous APCs.

25

The following abbreviations are used herein: T helper determinants - HD; Mean fluorescence intensity - MFI; Suppressogenic determinants - SD; Recombinant Tetanus Toxoid - rTT; tat-DR4 comprising residues 49-57 of HIV-1 tat and residues 64-88 of DRB1*0401 - Tat-DR4 chimeric peptide; T cell lines - TCL; T suppressor lymphocytes - Ts.

30

Materials and Methods

Peptide Synthesis and Ag Preparations

35

Recombinant Tetanus Toxoid (rTT) C fragment was obtained from Boehringer Mannheim (Indianapolis, IN) and conjugated to carboxylated polystyrene microparticles (Latex beads; Polysciences, Inc., Warrington, PA) using the Carbodiimide

-88-

Kit according to the manufacturer's instructions.

A chimeric peptide tat-DR4, comprising residues 49-57 of HIV-1 tat and residues 64-88 of DRB1*0401 was purchased from Chiron Technologies, Australia. The purity of the peptide was >85% as determined by reverse-phase HPLC. The amino acid sequence of this peptide is as follows:
RKKRRQRRRQKDLLEQKRAAVDTYCRHNYGVGES.

10 HLA Typing

Lymphocytes were typed for HLA class I antigens by conventional serology. The class II genotype of the cells was determined by genomic typing of *in vitro* amplified DNA with sequence-specific oligonucleotide probes for DRB1, DQA1, and DQB1. The HLA phenotype of the blood donor (PR) used in these studies is: HLA-A29, A32, B40, B44, DRB1*1101, DRB1*0701.

20 Generation of Antigen Specific T Cell Lines (TCL)

PBMCs from a healthy blood male (PR) were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding PBMCs at $2 \times 10^6/\text{ml}$ were stimulated in 24-well plates (Nunc, Inc., IL) with 10 $\mu\text{g}/\text{ml}$ of tat-DR4 peptide or with 50 μl (approximately 1mg) of rTT-beads in RPMI 1640 medium supplemented with 10% human serum (Sigma Chemical Co., St. Louis, MO), 2mM L-glutamine, and 50 $\mu\text{g}/\text{ml}$ gentamicin (Gibco, Grand Island, NY). On day five, 20 U/ml of rIL-2 (Boehringer Mannheim, Indianapolis, IN) were added. Ten days after priming, T cells ($2 \times 10^6/\text{ml}$) were collected, washed and restimulated with antigen in medium containing 20 U/ml of rIL-2 and irradiated (3000 rad) autologous PBMCs ($2 \times 10^6/\text{ml}$). Antigen specific T cell lines were obtained after two or three restimulations. Two cell lines, named PR-anti-rTT and PR-anti-tat-DR4 peptide were used in this study.

-89-

Cell Isolation and Culture

CD4⁺ and CD8⁺ T cells were isolated from TCL PR-anti-rTT by positive selection using Dynal CD4 and CD8 beads according
5 to the manufacturer's instructions. After 30 minutes of incubation at 4°C, the rosetted cells were washed and resuspended in 0.1ml of medium containing 15 microliters of DETACHaBead CD4/CD8. After 3 hours of incubation at 37°C, non-rosetted cells were collected, washed, and resuspended
10 in medium.

CD4⁺ and CD8⁺ T cells from TCL PR-anti-tat-DR4 peptide were separated by negative selection using Dynal CD4 and CD8 beads. CD8⁺CD28⁻ T cell suspensions were obtained from both
15 TCLs by depleting CD28⁺ T cells from purified CD8⁺ T cell suspensions. For this procedure goat-anti-mouse Dynal beads were coupled with mAb anti-CD28 (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions. The CD28-coupled beads were washed and incubated at 4 x 10⁷ beads per ml with 1 x 10⁷ CD8⁺ T cells for 20 min at 4°C with gentle end-over-end mixing. Non-rosetted cells were collected, washed and resuspended in complete RPMI 1640
20 medium.

25 The purity of the CD4⁺ and CD8⁺CD28⁻ suspensions used in blastogenesis assays was greater than 98% as indicated by cytofluorographic analysis.

Proliferation Assay

30 Antigen-specific CD4⁺ T cells or CD8⁺CD28⁻ T cells (30,000/well) obtained from TCLs were cultured with irradiated (3000 rad) autologous PBMCS, as APCs (30,000/well) in 96-well round bottom microplates (Nunc, Inc. Naperville, IL). T cell stimulation with rTT was accomplished using APCs which were pulsed with rTT (5 µg/ml) for 3 hours, then washed
35 and irradiated. tat-DR4 peptide was used at a concentration of 1 µM. CD8⁺CD28⁻ T cells were tested for suppressor

-90-

activity (30,000 cells/well) by addition to cultures containing Th cells (30,000/well) at the initiation of the blastogenesis assay. The cultures were labeled with [³H] TdR (0.5 m μ Ci/well) after 48 hours of incubation and harvested 18 hours later. [³H] TdR incorporation was then measured in a LK Betaplate liquid scintillation counter (Wallac, Inc., Gaithersburg, MD). Mean cpm of triplicate cultures and standard deviation of the mean were calculated. Standard deviations were less than 10% of the mean.

10

Antibody Blocking Assay

15

Monoclonal antibodies against HLA class I molecules were added to the cultures at the initiation of the proliferation assays. The human mAb OK4F9 (anti-HLA-A29), mAb OK3C8 (anti-HLA A32), mAb 13E12 (anti-HLA-B44) and mAb Ha2C10B12 (anti-HLA-B40) were used as cell culture supernatants. All antibodies were dialysed against RPMI 1640 medium before use.

20

TCR Spectratyping

25

Total RNA was extracted using QIAGEN columns (Qiagen Inc. Santa Clara, CA) from CD8⁺CD28⁻ Ts isolated from TCL PR-anti-rTT. RNA was reverse transcribed into cDNA using MMLV reverse transcriptase and primed with oligo (dT)₁₈ (Clontech Laboratories Inc., Palo Alto, CA) as recommended by the manufacturer. Aliquots of the cDNA synthesis reaction were amplified individually with 24 human Vb and the Cb primers that have been previously described (8,9). As an internal control for the amount of cDNA used for each Vb PCR reaction, a second reaction tube containing sense and antisense primers for the first exon of Cb region was included. A run-off reaction of the Vb-Cb PCR products was performed using a fluorochrome labeled Cb-specific primer. The size and fluorescence intensity of labeled run off products were then determined on a 377 DNA sequencer (Perkin Elmer Applied Biosystem Division, Foster City, CA) and

30

35

-91-

analyzed using the ABI PRISM 377 GENESCAN Analysis Program. The relative intensity of each Vb family was calculated as the peak area corresponding to each Vb family divided by the sum of all area peaks.

5

Flow Cytometry

T cell subsets were defined using mAb CD4-PerCP, CD8-FITC, and CD28-PE from Becton Dickinson, CA. Cell suspensions were phenotyped prior to use in blastogenesis assays using a FACScan flow cytometry instrument (Becton Dickinson, San Jose, CA) equipped with a 15mW Argon Laser. To study the expression of CD80, CD86, and CD40 on CD2 depleted PBMCS, i.e. CD20⁺ B cells, CD14⁺ monocytes and dendritic cells, used for antigen presentation, cells were incubated with saturating amounts of mAbs recognizing CD80-PE, CD20-FITC, CD14-FITC, CD86-PE. and CD40-PE (all from Becton Dickinson, San Jose, CA). CD20 positive and CD14 positive cells were gated in and analyzed for CD80, CD86, and CD40 expression. Five parameter analysis (forward scatter, side scatter and three fluorescence channels) were used for list mode data analysis. FL1 channel was used as fluorescence trigger, FL2 as analysis parameter. Mouse IgG (g1 and g2) reagents were used as isotype controls for nonspecific binding of test reagents and as markers for delineating the positive and negative populations. Calibrite flow cytometer beads (Becton Dickinson, San Jose, CA) and FACSCcomp program were used for calibration of the cytometer.

10
15
20
25

30

Results

Inhibition of Th reactivity to Tetanus Toxoid by Ts Cells

35

In previous studies it was demonstrated that xeno(6) and allospecific Ts recognize MHC-class I antigens on stimulating APCs (7). In order to direct soluble rTT protein to the endogenous antigen processing pathway, which supplies MHC-class I bound peptides, rTT was conjugated to microscopic

-92-

beads (10).

To determine whether the Th response to a nominal antigen is suppressed by autologous Ts, PBMCs from a healthy individual (PR) were primed *in vitro* with rTT conjugated beads. CD4⁺ Th and CD8⁺CD28⁻ Ts, were isolated from the resulting TCL (TCL PR-anti-rTT) and tested for reactivity to rTT in the presence of irradiated autologous PBMCs. CD4⁺ Th cells reacted vigorously to autologous APCs pulsed with rTT, while CD8⁺CD28⁻ T cells showed no proliferative response (Fig. 16).

When Th and Ts cells from TCL PR-anti-rTT were mixed together at a 1:1 ratio and primed with rTT, there was significantly less Th proliferation than in control cultures in which no Ts were added. The amount of inhibition seen in four repeat experiments ranged from 34 to 37% (Fig. 16). The inhibitory effect of Ts on Th reactivity to rTT was not due to competition for IL-2, as CD8⁺CD28⁻ Ts obtained from the allopeptide specific TCL (TCL PR anti-tat-DR4) caused no inhibition of Th reactivity to rTT (data not shown). Irradiation (3,000 rad) of CD8⁺CD28⁻ Ts cells prior to co-culture with Th and APCs showed no effect on suppressor activity, indicating that the suppressor cells are not radiation sensitive (data not shown).

25

Inhibition of Th reactivity to Tat-DR4 Peptide

Experimental evidence demonstrates that in monocyte/macrophage APCs there is communication between the exogenous and endogenous pathways of antigen processing and that antigens in the extracellular millieu can also be presented in association with MHC class I molecules (11). Recently, it was shown that the entry of exogenous proteins into the MHC class I pathway can be facilitated by conjugating proteins to a short cationic peptide derived from HIV-1 tat (residues 49-57) (12). Based on this knowledge a chimeric peptide consisting of residues 49-57 of tat and residues 64-88 of the DRB1*0401 molecule was synthesized. The latter

-93-

peptide, which corresponds to the third hypervariable region of the DRB1*0401 antigen, was previously shown to comprise the dominant epitope of the DR 0401 antigen recognized by T cells from individuals carrying the DR*1101 and DR*0701 alleles (13).

To achieve coexpression of Th and Ts epitopes on the same APCs the Tat-DR4 peptide was used for in vitro immunization of T cells from PR (who is DR1101/DR0701 heterozygous).

CD4⁺ and CD8⁺CD28⁻ T cells were isolated from TCL PR-anti-tat DR4 and tested alone or together for reactivity to peptide tat-DR4 presented by autologous APCs. Blastogenesis assays showed that peptide tat-DR4 stimulated the proliferation of CD4⁺ T cells but not of CD8⁺CD28⁻ T cells. In the presence of CD8⁺CD28⁻ T cells the response of CD4⁺ Th was suppressed by >70% in four repeat experiments (Fig. 17). CD8⁺CD28⁻ T cells from this line (PR-anti-tat-DR4) did not inhibit the reactivity to rTT of CD4⁺ T cells from TCL PR-anti-rTT. Similarly, CD8⁺CD28⁻ Ts cells obtained from TCL PR-anti-rTT did not inhibit the reactivity of Th from TCL PR-anti-tat-DR4 to tat-DR4, indicating that the suppressor effect is antigen-specific (data not shown).

To determine the MHC-restriction element required for peptide recognition by Ts blocking studies were performed using mAbs specific for the HLA-class I antigens expressed by PR (HLA A29, A32, B40 and B44). The blastogenic response of Th cells to peptide tat-DR4 was of the same order of magnitude in cultures with and without anti-HLA class I mAbs (Fig. 18A). Ts cells inhibited the reactivity of Th cells to the peptide in cultures without mAbs or with mAbs to HLA-A29 or B40. Hence, blocking of HLA-A29 or B40 by mAbs did not prevent Ts activation, indicating that HLA-A29 and B40 molecules did not present suppressogenic determinants of peptide tat-DR4. The suppressor effect, however, was completely abolished when mAbs specific for HLA-A32 or B44 were added to the assay (Fig. 18B) This indicates that the peptide(s) recognized by

-94-

Ts are presented by HLA-A32 and B44.

Spectratyping of Vb Genes Expressed by CD8⁺CD28⁻ Ts from TCR PR-Anti-TT

5 Study of the TCR-Vb gene repertoire expressed by xenospecific Ts showed an oligoclonal expansion of a few Vb families. (6) To determine whether the Vb repertoire used by rTT-specific Ts is also restricted the expression of Vb families in the population of CD8⁺CD28⁻ Ts derived from TCL PR-anti-rTT was analyzed. CD8⁺CD28⁻ Ts were sorted from the TCL after two stimulations with rTT, and then expanded, by weekly restimulation with rTT-pulsed APCs, in IL-2-containing medium. Blastogenesis assays using Th and Ts showed that the CD8⁺CD28⁻ population maintained suppressor activity throughout five consecutive cycles of antigenic stimulation. T cells obtained at later times showed no suppressor activity when co-cultured with CD4⁺ Th.

10

15

20 Analysis of the TCRs expressed by suppressor cells propagated for five weeks in culture showed that Vb3, Vb5, Vb13, Vb15, and Vb19 were expressed with a relative intensity greater than 10%. The other Vb families were either completely absent (such as Vb6, Vb8, Vb10, Vb11, Vb18, Vb20, and Vb21), or expressed with a relative intensity lower than 5%. After an additional week of expansion, the CD8⁺CD28⁻ population lost suppressor activity and expressed only the Vb3 and Vb5 families (Fig. 19). The loss of Vb13, Vb15 and Vb19 coincided with the loss of suppressor activity suggesting that they represented the Ts population.

25

30

35 Spectratyping of the TCRs used by CD4⁺ Th cells from TCL PR-anti-rTT showed expression of V β 3, V β 5, V β 9, V β 10, and V β 11. Two of the V β families (V β 3 and V β 5) represented in the CD4⁺ T cell population, were also found within the CD8⁺CD28⁻ population (where they apparently had no Ts function). Usage of V β 9, V β 10, and V β 11 was unique to rTT-specific Th cells. This finding suggests that the

-95-

determinants recognized by Th and Ts on the rTT molecule are distinct.

5 Effect of Antigen Specific Ts on the Expression of CD80, CD86, and CD40 on APCs.

In previous studies it was shown that allospecific Ts downregulate the expression of CD80 and CD86 on the APCs used for priming, interfering with the delivery of costimulatory signals required for Th activation (7). To establish whether such a mechanism is also involved in Ts-induced-downregulation of Th responses to nominal antigens analysed was the expression of CD80, CD86, and CD40 on antigen-pulsed APCs, cultured for 24 hours in the presence, 10 or absence of Ts.

15 For these experiments CD4⁺ Th (5×10^5) were mixed with CD8⁻CD28⁻ Ts (1.5×10^6) and APCs (5×10^5) in medium containing tat-DR4 peptide (1 mM). Control cultures containing only APCs or APCs and Ts were set up in parallel. Within 24 hours the expression of CD40 on APCs increased dramatically in the presence of Th as indicated by the shift of the Mean Fluorescence Intensity (MFI) from 633 to 2230, 20 in cultures without, and with Th respectively. However, when Th were co-cultured with Ts, the level of CD40 expression on APCs was about 50% lower (MFI of 1114) than in the absence of Ts (Fig. 20).

25 Similarly, the level of CD80 and CD86 expression on APCs was greatly enhanced by Th, while Ts induced only a slight elevation of CD80 and CD86 expression. When Ts were added to Th cultures, the expression of CD80 and CD86 remained at the level seen in the absence of Th (Fig. 20). This indicates that Ts interfere with the Th-induced upregulation 30 of costimulatory molecules (CD40, CD80, and CD86) on APCs.

35 Discussions

-96-

Two general approaches to antigen-specific immunotherapy have been proposed (2). The first is to block the activation of T helper cells using MHC blocking peptides, tolerogenic concentrations of antigen or TCR antagonists (2). The second approach involves the induction of antigen-specific regulatory T cells which downregulate immune responses at inflammatory sites (2). Although T suppressor cells have been induced in a variety of experimental models their role and mechanism of action are not well understood. In both mouse and human systems, suppressor cells have been shown to derive from CD4 as well as CD8 subsets (1-5). It has been suggested that CD4⁺ T cells act as suppressor inducers, while CD8⁺ T cells act as suppressor effectors (1). A number of cytokines, including interferons, prostaglandins, tumor necrosis factor, transforming growth factors, and interleukin-10 were shown to exert suppressive activity on the growth, differentiation and effector functions of T lymphocytes (14-16).

Suppression generated by oral tolerance to certain autoantigens is antigen and disease specific. This observation suggests that the secretion and action of cytokines must occur in the microenvironment where the immune response is stimulated (2, 14, 15).

Although suppressogenic determinants (SD) of well-defined antigenic proteins have been shown to be different from immunogenic T helper determinants (HD) it has been suggested that they must be localized on a single processed antigenic fragment (1, 17-19) and/or presented by the same APC in a multi-cell cluster for suppression to occur (20, 21).

The concept that the interaction between Ts and Th cells is regional in nature and requires proximity of suppressogenic and immunogenic determinants, is also supported by previous studies from our laboratory showing that in the allogeneic and xenogeneic system, suppression requires an antigen-mediated tripartite interaction between Ts, Th and

-97-

APCs. It was demonstrated that suppression is specific for MHC-class I/peptide complexes expressed by the APC used for priming and results in diminished T helper cell reactivity to MHC-class II antigens co-expressed by the same APCs (7).
5 This effect was caused by suboptimal costimulation of alloreactive or xenoreactive CD4⁺ Th in the presence of CD8⁺CD28⁻ Ts. Hence Ts induce downregulation or inhibit upregulation of B7 molecules (CD80 and CD86) on priming APCs and of CD154 (CD40L) on activated Th. Cell-mixture experiments in which Ts and Th cells were co-cultured with two different APCs, one expressing the MHC-class I antigens recognized by Ts, and the other, the MHC-class II antigens recognized by Th, showed no suppression indicating that SD and HD must be presented by the same APC to reveal suppression (6,7).

10
15
20
25
The present study demonstrates that Th recognition of nominal antigens is subjected to the same mechanism of down-regulation by MHC-class I restricted CD8⁺CD28⁻ cells. Study of the TCR-repertoire used by rTT-specific Th and Ts cells showed overlap of only two TCR Vb families (Vb3 and Vb5), neither of which seemed to contribute to the suppressor activity of the CD8⁺CD28⁻ population. This lack of overlap between the Vb repertoire used by rTT-specific Th and Ts is consistant with the restriction of CD4⁺ Th cells to MHC class II and CD8⁺ Ts cells to MHC class I molecules and with the hypothesis that HD and SD determinants are distinct (1).

30
Additionally, the oligoclonality of the rTT-specific Th and Ts populations suggests that, in spite of its complexity, the TT molecule has a limited number of immunogenic and suppressogenic determinants recognized by T cells in vitro.

35
To explore the possibility that exogenous antigens can be used for induction of MHC-class I restricted T suppressor cells a chimeric peptide consisting of residues 49-57 of HIV-tat, which facilitates entry into the endogenous antigen processing pathway, and residues 64-88 of the DR 0401

-98-

molecule was used. Processing of the chimeric tat-DR4 peptide by APCs resulted in presentation of both Ts and Th determinants. Ts generated in this system inhibited Th proliferation in an antigen-specific manner. Blocking studies using monoclonal anti-HLA-class I antibodies, demonstrated that the determinant(s) recognized by Ts were presented by two of the responder's HLA class I molecules (HLA-A32 or B40). Since the binding motifs of these two HLA class I molecules differs it is likely that the determinants which CD8⁺CD28⁻ T cells recognize on each of them are not the same. Similarly the amino-acid residues of the tat-DR4 peptide which contact the TCR of Th and Ts cells are probably different due to structural differences between MHC-class I and class II molecules. However, it remains to be studied whether differences in peptide presentation by MHC class I and class II antigens explain the differences in the TCR repertoire and in the selection of suppressogenic and immunogenic determinants.

Cytofluorometric analysis of CD40, CD80, and CD86 molecules on APCs co-cultured with Th and Ts showed that the upregulatory effect exhibited by Th on the expression of costimulatory molecules is drastically inhibited in the presence of Ts. Although the mechanism accounting for the effect of Ts on APCs is still under study, these data suggest that inhibition of Th proliferation is secondary to downregulation of the costimulatory potential of APCs. The finding that Ts downregulate the expression of CD40 on APCs during the tripartite interaction with Th is particularly important in light of the recent demonstration that CD40 is also required for the tripartite interaction between Th, cytotoxic T cell lines (Tc), and APC (22-25).

The finding presented here that Ts cells which inhibit Th reactivity to allo and xenogeneic cells as well as Th reactivity to protein antigens can be educated *in vitro* has significant implications for the development of antigen specific therapy for treatment of allograft rejection and

-99-

autoimmune diseases. It is conceivable that autologous Ts generated in vitro can be used for "adoptive" transfer of suppression. Alternatively, active immunization with suppressogenic peptides, targeted to the endogenous pathway 5 may offer a viable strategy for inhibition of indirect allorecognition, a major contributor to rejection, and of autoimmune diseases.

-100-

References for the Third Series of Experiments

1. Sercarz EE, Krzych U: The distinctive specificity of antigen-specific suppressor T cells. Immunol Today 12:111, 1991.
- 5
2. Hafler DA, Weiner HL: Immunologic mechanisms and therapy. Immunol Rev 144:75, 1995.
- 10
3. Roser BJ: Cellular mechanisms in neonatal and adult tolerance. Immunol Rev 107:179, 1989.
- 15
4. Salgame P, Abrams JS, Clayberger C, Goldstein H, Convit J, Modlin RL, Bloom BR: Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science 254:279, 1991.
- 20
5. Liblau R, Tisch R, Bercovici N, McDevitt HO: Systemic antigen in the treatment of T-cell-mediated autoimmune diseases. Immunol Today 18:599, 1997.
- 25
6. Ciubotariu R., A.I. Colovai, G. Pennesi, Z. Liu, D. Smith, P. Berlocco, R. Cortesini, and N. Suciu-Foca: Specific suppression of human CD4⁺ T helper cell responses to pig MHC antigens by CD8⁺CD28⁻ regulatory T cells. J. Immunology 161(10):5193-5202., 1998.
- 30
7. Liu Z, Tugulea S, Cortesini R, Suciu-Foca N: Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8⁺CD28⁻ T cells. Int Immunol 10(6):775-783, 1998.
- 35
8. Puisieux I, Even J, Pannetier C, Joterau F, Favrot M, Kourilsky P: Oligoclonality of tumor-infiltrating lymphocytes from human melanomas. J Immunol 153:2807, 1994.
9. Gardevet L, Dulphy N, Dovay N, Chalumeau N, Schaeffer V, Zilber MT, Lim A, Even J, Mooney N, Gelin C, Gluckman E,

-101-

Charron D, Toubert A: The umbilical cord blood ab T-cell repertoire: characteristics of a polyclonal and naive but completely formed repertoire. Blood 91:340, 1998.

5 10. Harding CV, Song R: Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. J Immunol 153:4925, 1994.

10 11. Harris PE, Colovai AI, Maffei A, Liu Z, Suciu-Foca N: Major histocompatibility complex class I presentation of exogenous and endogenous protein-derived peptides by a transfected human monocyte cell line. Immunol 86:606, 1995.

15 12. Kim DT, Mitchell DJ, Brockstedt DG, Fong L, Nolan GP, Fathman CG, Engleman EG, Rothbard JB: Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. J Immunol 159:1666, 1997.

20 13. Liu Z, Harris P, Colovai AI, Reed EF, Maffei A, Suciu-Foca N: Suppression of the indirect pathway of T cell reactivity by high doses of allopeptide. Autoimmunity 21:173, 1995.

25 14. Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL: Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor b after antigen-specific triggering. Proc.Natl Acad Sci 89:421, 1992.

30 15. Lider O, Santos LMB, Lee CSY, Higgins PJ, Weiner HL: Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. J Immunol 142:748, 1989.

35 16. MacAry PA, Holmes BJ, Kemeny DM: Ovalbumin-specific, MHC class I-restricted, ab-positive, Tc1 and Tc0 CD8⁺ T cell

-102-

clones mediate the in vivo inhibition of Rat IgE. J Immunol 160:580, 1998.

5 17. Shivakumar S, Sercarz EE, Krzych U: The molecular context of determinants within the priming antigen establishes a hierarchy of T cell induction: T cell specificities induced by peptides of β -galactosidase vs. the whole antigen. Eur J Immunol 19:681, 1989.

10 18. Krzych U, Fowler AV, Sercarz EE: Repertoires of T cell directed against a large protein antigen, β -galactosidase: Only certain T helper or T suppressor cells are relevant in particular regulatory interactions. J Exp Med 162:311, 1985.

15 19. Krzych U, Fowler AV, Miller A, Sercarz EE: Repertoires of T cells directed against a large protein antigen, β -galactosidase: Helper cells share a more restricted specificity repertoire than proliferative cells. J Immunol 128:1529, 1982.

20 20. Asano Y, Hodes RJ: T cell regulation of B cell activation: An antigen-mediated tripartite interaction of Ts cells, Th cells, and B cells is required for suppression. J Immunol 133:2864, 1984.

25 21. Mitchison NA, O'Malley C: Three-cell-type clusters of T cells with antigen-presenting cells best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. Eur J Immunol 17:1579 1987.

30 22. Lanzavecchia A: License to kill. Nature 393:413, 1998.

35 23. Ridge JP, Di Rosa F, Matzinger P: A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and T-killer cell. Nature 393:474, 1998.

-103-

24. Bennett SRM, Carbone FR, Karamalis F, Flavells RA, Miller JFAP, Heath WR: Help for cytotoxic-T-cell responses is mediated by CD40 signaling. Nature 393:478, 1998.

5 25. Schoenberger SP, Toes REM, van der Voort EIH, Offringa R, Melief CJM: T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 393:480, 1998.

10

Fourth Series of Experiments**Inhibition of CD40 Signaling Pathway in Antigen Presenting Cells by T Suppressor Cells**

CD8⁺ T lymphocytes contain precursors of cytotoxic and suppressor cells [1]. The generation of Tc can occur in "sequential two cell interactions"; first, between CD4⁺ Th cells and APCs and next, between activated APCs and CD8⁺ Tc cells [2-4]. Another population of CD8⁺ T cells, which lack the potential of becoming Tc, is characterized by the ability to display antigen specific T suppressor function [1, 5-7]. It has been previously shown that this population of Ts derives from the oligoclonal CD8⁺ CD28⁻ subset [8] and recognizes MHC class I/peptide complexes on the cell surface of APCs [5-7].

Allospecific Th and Ts were generated by multiple priming of human T cells with irradiated APCs from MHC class I and class II disparate blood donors. In this system, CD4⁺ Th, isolated from the T cell line, recognize specifically MHC-class II antigens expressed by the APCs used for priming. CD8⁺ CD28⁻ T cells from the same TCL inhibit, in a dose-dependent manner, the proliferative response of CD4⁺ Th. Inhibition of Th proliferation is not associated with killing of either APCs or CD4⁺ Th. CD8⁺ CD28⁻ T cells recognize specifically MHC class I antigens expressed by the stimulating APCs and render them unable to upregulate the expression of CD80 and CD86 in the presence of Th. This inhibitory effect requires cell-to-cell interactions between Th, Ts and the APCs used for priming [5].

The aim of the present study was to investigate whether the suppressor effect requires the concomitant interaction between Ts, Th and APCs or sequential two cell interactions (first, between Ts and APCs and next, between "suppressed" APCs and Th) and whether it is mediated by inhibition of the CD40-signaling pathway.

-105-

MATERIALS AND METHODS**Generation and Isolation of allospecific CD4⁺ and CD8⁺CD28⁻ T Cell**

5 Peripheral blood mononuclear cells from healthy blood volunteers were primed in MLC with irradiated (1600 rad) PBMCs from MHC-mismatched blood donors. Stimulating cells from the allogeneic donor were depleted of T cells using CD2 magnetic beads (Dynal, New York, NY). After seven days of 10 incubation in complete medium) RPMI 1640 with 10% human serum 2mM L-glutamine and 50µg/ml gentamycin) (Gibco, Grand Island, NY), responding T cells were restimulated with irradiated APCs (CD2-depleted PBMC) from the same blood donor. Three 15 days later CD4⁺ T cells were isolated by positive selection with Dynal CD4 beads and Detachabeads, according to the manufacturer's instructions. CD8⁺CD28⁻ T cells, from the same culture, were purified first by positive selection of CD8⁺ T cells with Dynal CD8 beads and Detachabeads, and then by negative selection of CD28⁻ cells using anti-CD28 mAb 20 coupled to Dynal beads. The separated cells were cultured in complete medium supplemented with 10 µ/ml rIL-2 (Boehringer Manneheim, Indianapolis, IN) for four more days. CD4⁺ Th 25 cells and CD8⁺CD28⁻ Ts cells were further propagated in IL-2 containing medium by weekly stimulations with irradiated APCs from the same blood donor. Prior to testing, CD4⁺ T cells were purified one more time by negative depletion of CD8 cells using CD8 beads. Similarly, CD8⁺CD28⁻ T cells were 30 repurified by depletion of CD28⁻ T cells using CD28 beads. The purity of CD4⁺ Th and CD8⁺CD28⁻ Ts was determined by flow cytometry as previously described [5-7].

Proliferation Assays

35 CD4⁺ T cells (5×10^4 /well) from T cell lines were tested for reactivity to irradiated allogeneic APC (2.5×10^4 /well) in the presence or absence of CD8⁺CD28⁻ T cells (2.5×10^4 /well) from the same TCL. When CD3 antibody was used for T cell

-106-

activation, the plates were coated overnight with CD3 mAb (1g/ml), then blocked with complete medium and washed. CD4⁺ and CD8⁺CD28⁺ T cells were used at 5 X 10⁴/well. Cultures were pulsed with ³H thymidine after 48 h of incubation and harvested 18 h later. ³H thymidine incorporation was measured in an LK Betaplate counter. Mean cpm of the triplicate cultures and SD to the mean were calculated.

10 Suppression of CD40L Expression on Activated The Cells

15 Allospecific Th cells were cultured with allogeneic APCs, in the presence or absence of Ts cells for 6 h. mAb CD40L (1g/ml) was added to the culture medium to prevent the rapid internalization of CD40L molecules on the surface of CD4⁺ T cells [9]. The suspension was washed, stained with FITC-conjugated goat-anti-mouse Ig (Becton Dickinson, Mountain View, CA), then washed and stained with CD4-PE (Becton Dickinson). Four parameter analyses (forward scatter, side scatter and two fluorescence channels) were used for list mode data analysis. Mouse IgG (γ 1 and γ 2) was used as isotype control for non-specific binding of test reagents and as markers for delineating the positive and negative populations. CaliBRITE flow cytometry beads (Becton Dickinson) and FACSComp program were used for calibration of the FACScan flow cytometry instrument (Becton Dickinson).

20 25 The expression of CD40L on Th activated by use of mAb anti-CD3, in the presence or absence of Ts, was also analyzed after 6 h of incubation by staining the cells with CD40L-PE and CD4-FITC (Becton Dickinson).

30 Suppression of costimulatory molecules expressed by APC

35 Crosslinking of CD40 molecules on APCs was accomplished by incubating CD2-depleted PBMCs at 1 X 10⁶/ml with an equal number of cells from the CD40L⁺ D1.1 line [10]. Allospecific CD8⁺ CD28⁺ Ts (1 X 10⁶/ml) primed *in vitro* to the same APCs

-107-

were added to parallel cultures. After 24 h of incubation cells were washed, stained and analyzed for expression of costimulatory molecules. The second method for CD40 crosslinking consisted of incubating APCs ($2 \times 10^6/\text{ml}$) with FcRII CD32⁻ L cells ($0.5 \times 10^6/\text{ml}$), in medium containing mAb CD40 G28-5 (100ng/ml) and rIL-4 (10 ng/ml) (Boehringer Mannheim). Cultures were set up in parallel with and without allospecific Ts ($2 \times 10^6/\text{ml}$). After 48 h of incubation cultures were washed and processed for cytofluorometric analysis.

Aliquots of the same cultures were stained simultaneously with CD20-FITC, CD14-FITC and PE-conjugated mAb specific for one of the following markers: CD40, CD54, CD58, CD80 and CD86 (Pharmingen, San Diego, CA).

To study the kinetics of Ts-mediated suppression of costimulatory molecules induced by the Th, APCs ($1 \times 10^6/\text{ml}$) were incubated 48 h with allospecific CD4⁺ Th cells ($2 \times 10^6/\text{ml}$). CD8⁺CD28⁻ Ts ($1 \times 10^6/\text{ml}$) from the same TCL were added to the culture at the initiation of the assay or 6 and 18 h later. At the end of the incubation time (48 h), cells were washed and stained with CD20-FITC, CD14-FITC, and CD40, CD54, CD58, CD80, or CD86 PE, as described above.

25 RESULTS AND DISCUSSION

To define the cellular interactions mediating the suppressor activity of CD8⁺CD28⁻ Ts, the effects of these cells on the earliest events occurring during the program of CD4⁺ Th activation were studied. An early and critical step in Th activation is the expression of CD40L (CD154) [11, 12]. This molecule interacts with CD40 on APCs and induces APCs to upregulate surface CD80 and CD86 molecules [10, 13, 14].

35 It is possible that Ts act directly on Th, inhibiting the expression of CD40L or, alternatively, they may act on APCs, blocking the CD40 signaling pathway. To discriminate between these two possibilities, first determined was whether Ts can

-108-

inhibit Th in the absence of APCs. Experiments in which allospecific Th and Ts were co-cultured in the presence of mAb anti-CD3 showed that Ts do not inhibit Th proliferation or CD40L expression (Figs. 21A, 21B). In contrast, when allospecific Th and Ts are cultured together with the APCs used for priming, both the expression of CD40L and the proliferative capacity of Th are inhibited (Figs. 21C, 21D). These results indicate that the suppressive activity of Ts on Th proliferation is not determined by the direct interaction between Ts and Th and that it requires the presence of APCs. This finding is consistent with the previous observation that Ts and Th must recognize the same APC for suppression to occur [5, 6]. It is, therefore, possible that whether APCs can or cannot activate Th depends on their previous encounter with either CD4⁺Th or CD8⁺CD28⁺Ts.

To explore this possibility, Ts were added to cultures containing allospecific Th and the APCs used for priming, 0, 6 and 18 h after the initiation of the assay. The expression of CD40, CD54, CD58, CD80 and CD86 on APCs was analyzed 48 h, and Th proliferation was measured 72 h, after the initiation of the cultures. In the absence of Ts, Th show strong proliferation in response to stimulation with APCs (Fig. 22) and induce the upregulation of CD54, CD58, CD80 and CD86 on APCs (Fig. 23). In contrast, when Ts were added to the cultures at time 0, Th proliferation was strongly inhibited (Fig. 22) and the level of CD54, CD58, CD80 and CD86 expression on APCs was greatly diminished (Fig. 23). The inhibitory effect of Ts decreased when they were added 6 h after initiation of cultures and was virtually absent when added 18 h later (Fig. 22 and Fig. 23). Taken together these data indicate that suppression is an early event which requires the presence of APCs and that APCs may activate CD4⁺ Th only if they have not first interacted with Ts.

To determine whether Ts render the APCs unable to stimulate Th, allospecific Ts were incubated with the APCs used for priming. After 6 to 24 h of incubation, Ts were removed from

-109-

the cultures and the APCs were used for stimulating Th derived from the same TCL. While APCs incubated with naive CD8⁺CD28⁺ T cells elicit strong proliferation of Th, APCs that have been incubated with allospecific Ts for 6 to 24 h fail to trigger Th blastogenesis in 3-day assays (Table 7). Propidium iodine and Annexin V staining of the APCs exposed to Ts for 24 h show no loss of viability [5, 6], indicating that Ts do not kill the APCs, yet convert them to a state in which they are no longer capable of sustaining the activation of Th. These data indicate that the costimulatory capacity of APCs is altered by a prior encounter with Ts.

10

RECEIVED
U.S. PATENT AND TRADEMARK OFFICE
JULY 10 2002

Table 7

Ts render APCs unable to stimulate the proliferation of allospecific Th (^{3}H thymidine incorporation)

Cultures	Self-APC	Medium	Allogeneic APCs preincubated			Allospecific CD'28 [*] Ts cells		
			Naive CD8'28 [*] T cell			Ts cells		
			6h	24h	6h	24h	6h	24h
Th	1,141 ± 67*	40,283 ± 2,941	42,784 ± 3,541	41,965 ± 3,762	44,283 ± 4,023	5,980 ± 548	418±36	
Ts	470 ± 45	470 ± 45	532 ± 49	366 ± 41	1,324 ± 142	456 ± 43	64 ± 55	
Th + Ts	936 ± 89	7,936 ± 689	8,934 ± 815	6,009 ± 524	7,025 ± 673	2,712 ± 231	86 ± 11	

*Mean CPM ± SD.

Th or/and Ts from an allospecific TCL were incubated for 3 days with autologous or allogeneic APCs (CD2⁺ depleted PBMC). The allogeneic APCs used for stimulation were preincubated for 6 or 24 h with allospecific CD8'28^{*} cells. The U cells were then depleted from the mixture with CD2 magnetic beads. The cultures were labeled and harvested after 72 h. Results are representative of three different experiments.

-111-

Since ligation of CD40 molecules on APCs has been shown to upregulate the expression of costimulatory molecules [10, 13], it is possible that Ts can inhibit the CD40 signaling pathway. To explore this hypothesis two well established systems were used. In one of these systems, upregulation of costimulatory molecules on APCs through CD40 signaling is accomplished by ligation of CD40 molecules on APCs using the D1.1 Jurkat T cell line, that constitutively expresses CD40L [10]. In the other system, CD40 antibody bound to the Fc (CD32) receptor of L cells were added to APCs in cultures containing IL-4 [13]. Crosslinking of CD40 molecules on APCs either by incubation for 24 h with the CD40L positive D1.1 line or by incubation for 48 h with anti-CD40 antibody results in upregulation of CD54, CD58, CD80 and CD86 molecules on APCs (Table 8). CD40 triggering also increases the mean fluorescence intensity (MFI) of CD40 on APCs (Table 8). In contrast, in the presence of allospecific Ts, APCs cultured for the same periods of time with D1.1 T cell line or CD40 antibody failed to upregulate any of these molecules (CD40, CD54, CD58, CD80 and CD86). The data indicate that Ts inhibit the costimulatory capacity of APCs, by interfering with CD40 dependent signaling.

Table 8
T_s inhibit CD40-induced upregulation of costimulatory molecules on APCs

Cultures	%Positive APCs (MFI*)			
	CD40	CD54	CD58	CD80
APC	99(203)	60(350)	42(374)	17(90)
APC + D1.1	99(312)	85(358)	70(380)	41(141)
APC + D1.1 + T _s	99(172)	11(147)	14(209)	7(78)
APC	99(231)	65(283)	40(315)	16(78)
APC + CD 40Ab + IL-4	99(430)	97(443)	80(458)	31(109)
APC + CD 40Ab + IL-4 + T _s	93(201)	56(192)	22(263)	10(47)
				33(221)

* Mean channel fluorescence intensity.
 APCs (CD2⁻ cell-depleted PBMC) were cultured with either CD40L⁺ D1.1 cells or mAb CD40 bound to Fc_Y receptor of L cells plus IL-4. T_s were added at the initiation of the assay. Expression of costimulatory molecules on CD14⁺ and CD 20⁺ APC was analysed by flow cytometry. Results are representative of three independent experiments.

O f

-113-

APCs (CD2⁻ cell-depleted PBMC) were cultured with either CD40L⁻ D1.1 cells or mAb CD40 bound to Fey receptor of L cells plus IL-4. Ts were added at the initiation of the assay. Expression of costimulatory molecules on CD14⁺ and CD 20⁺.
5 APC was analysed by flow cytometry. Results are representative of three independent experiments.

Taken together these data indicate that Ts interacts directly with APCs, inhibiting CD40-mediated CD80 and CD86 upregulation. The "suppressed" APCs are rendered unable to induce and sustain the full program of Th activation. Thus, Th exposed to "suppressed" APCs fail to upregulate completely CD40L expression due to an aborted "crosstalk", that is normally based on CD40-mediated upregulation of CD80 and CD86
10 [15, 16].

There is ample evidence that in the absence of co-stimulation TCR interaction with MHC/antigen complexes can lead to T cell anergy [17-19]. T cell anergy can be restored by crosslinking the CD28 molecule or by the use of exogenous IL-2 [17-19]. It was found that addition of rIL-2 to cultures containing Th, Ts and APC restores Th proliferation (Fig. 24). This indicates that Th are rendered anergic by Ts-treated APCs consistent with the previous finding that CD28 crosslinking restores Th reactivity in cultures with Ts [5].
20
25

The data herein support a model in which T-cell mediated suppression can result from the sequential interaction between first, TS and APCs and next, "suppressed" APCs and Th (Fig. 25). In this regard the present findings confirm and extend the "temporal bridging" model recently described to account for the complex role that APCs play in Th-mediated generation of CD8⁺ Tc[2-4]. Furthermore, the present data complement the finding that CD40 signaling is essential for conditioning APCs, by demonstrating that Ts inhibit this pathway. New data show that Ts inhibit The-induced activation of NF-B in APC, thus interfering with the
30
35

-114-

upregulation of B7 costimulatory molecules (Li, J., Liu, Z., Jiang, S., Cortesini, R., Lederman, S., Suciu-Foca, N. submitted).

5 Further dissection of the molecular interaction between Ts and APCs should allow the development of new strategies for specific suppression of the immune response in transplantation and autoimmune diseases.

10

The document contains 10 pages in total.

-115-

References for the Fourth Series of Experiments

1. Damie NK, Mohagheghpour N, Hansen JA, Engleman EG: Alloantigen-specific cytotoxic and suppressor T lymphocytes are derived from phenotypically distinct precursors. *J Immunol* 131:2296, 1983.
2. Ridge JP, Di Rosa F, Marzinger P: A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature* 393:474, 1998.
3. Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR: Help for cytotoxic-T-Cell responses is mediated by CD40 signaling. *Nature* 393:478, 1998.
4. Schoenberger SP, Toes REM, van der Voort EIH, Offringa R, Melief CJM: T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480, 1998.
5. Liu Z, Tugulea S, Cortesini R, Suciu-Foca N: Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8⁺CD28⁺ T cells. *Int Immunol* 10:775, 1998.
6. Ciubotariu R, Colovai AI, Pennesi G, Liu Z, Smith D, Berlocco P, Cortesini R, Suciu-Foca N: Specific suppression of human CD4⁺ Th cell responses to pig MHC antigens by CD8⁺CD28⁺ regulatory T cells. *J Immunol* 161:5193, 1998.
7. Jiang S, Tugulea S, Pennesi G, Liu Z, Mulder A, Lederman S, Harris P, Cortesini R, Suciu-Foca N: Induction of MHC-class I restricted human suppressor T cells by peptide priming *in vitro*. *Hum Immunol* 59:690, 1998.

-116-

8. Monteiro J, Batliwalla F, Ostrer H, Gregersen PK: Shortened telomeres in clonally expanded CD28⁻CD8⁻ T cells imply a replicative history that is distinct from their CD28⁺CD8⁻ counterparts. *J Immunol* 156:3587, 1996.

5

9. Roy M, Aruffo A, Ledbetter J, Linsley P, Kehry M, Noelle R: Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. *Eur J Immunol* 25:596, 1995.

10

10. Yellin MJ, Sinning J, Covey LR, Sherman W, Lee JJ, Glickman-Nir E, Sippel KC, Rogers J, Cleary AM, Parker M, Chess L, Lederman S: T lymphocyte T cell-B cell-activating molecule/CD40-L molecules induce normal B cells or chronic lymphocytic leukemia B cells to express CD80 (B7/BB-1) and enhance their costimulatory activity. *J Immunol* 153:666, 1994.

15

20. Lederman S, Yellin MJ, Krichevsky A, Belko J, Lee JJ, Chess L: Identification of a novel surface protein on activated CD4⁺ T cells that induces contact-dependent B cell differentiation (help). *J Exp Med* 175:1091, 1992.

25

12. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Atuffo A: A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci* 89:6550, 1992.

30

13. Ranheim EA, Kipps TJ: Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med* 177:925, 1993.

35

14. Goldstein MD, Debenedette MA, Hollenbaugh D, Watts TH: Induction of costimulatory molecules B7-1 and B7-

-117-

2 in murine B cells. The CBA/N mouse reveals a role for Bruton's tyrosine kinase in CD40-mediated B7 induction. Mol Imm 33:541, 1996.

5 15. Clark EA, Ledbetter JA: How B and T cells talk to each other. Nature 367:425, 1994.

10 16. Banchereau J, Steinman RM: Dendritic cells and the control of immunity. Nature 392:245, 1998.

15 17. Jenkins MK, Schwartz RH: Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. J Exp Med 165:302, 1987.

20 18. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP: CD28-mediated signalling co-stimulates murine T cells and prevents induction of energy in T-cell clones. Nature 356:607, 1992.

25 19. Schwartz RH: Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in Interleukin-2 production and immunotherapy. Cell 71:1065, 1992.

30

35

-118-

Fifth Series of Experiments

In the first through fourth series of experiments, we identified and characterized human antigen specific T suppressor cells (Ts). It was shown that Ts inhibits the costimulatory activity of APC blocking NF- κ B activation and transcription of costimulatory molecules. To explore the underlying mechanism we used for allostimulating peripheral blood B cells or cells from the dendritic cell line KG-1. Total RNA prepared from KG-1 or from B cells that have been exposed to allospecific Th, Ts or Th/Tz mixtures for 12 hours was used in a cDNA micro-array system to identify genes which are differentially expressed in APC. Although transcription of a wide array of genes was suppressed, expression of 10-15 genes was up-regulated >2-3 fold in APC cocultured for 12 hours with Ts or Ts/Th mixtures. Included in this latter group are the Monocyte Inhibitory Receptor (MIR-10 or ILT4), ILT2 (MIR7), and ILT3. MIR-10, MIR7 (ILT2) and ILT3 belong to a family of leukocyte inhibitory receptors (LIRs) which bear homology to killer inhibitory receptors (KIRs). These molecules interact with MHC-class I molecules via Ig-like domains and regulate negatively the activation of APC, recruiting an inhibitory signaling molecule, tyrosine phosphatase SHP-1. These data indicate that Ts-induced suppression of APC is based on an active mechanism by up-regulating the expression of a class of inhibitory receptors which transmit negative inhibitory signals in APC. Ts provides an essential regulatory mechanism through which immune tolerance can be achieved.

The fifth series of experiments studies the function of MIR-genes which mediate the T effect on APC. Upregulation of MIR expression renders the APC tolerogenic as they induce Th anergy, rather than Th stimulation. Hence, overexpression of MIRs in APC provides a therapeutic tool for induction of tolerance.

The fifth series of experiments studies the function of MIR-

-119-

genes which mediate the T effect on APC. Upregulation of MIR expression renders the APC tolerogenic as they induce Th anergy, rather than Th stimulation. Hence, overexpression of MIRs in APC provides a therapeutic tool for induction of tolerance.

5

10

15

20

25

30

35

-120-

What is claimed is:

1. A method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises:

5

a) obtaining peripheral blood T cells from a subject;

10

b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line;

15

c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b);

20

d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c);
25
e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and

30

f) expanding in culture the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.
35

-121-

2. The method of claim 1 wherein the MHC class I antigen is an HLA-A or HLA-B antigen expressed by the APC used for priming in step (b).

5 3. The method of claim 1 wherein the MHC class II antigen is an HLA-DR, HLA-DQ or HLA-DP antigen.

10 4. Antigen specific allospecific human suppressor CD8+ CD28+ T cells produced by the method of claim 1.

15 5. A method of generating xenospecific human suppressor CD8+CD28- T cells which comprises:

a) obtaining peripheral blood T cells from a human subject;

20 b) stimulating by multiple priming a human T cell line from the T cells obtained in step (a) with a xenogeneic antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen;

25 c) isolating primed human CD8+ T cells and human_CD4+ T helper cells from the T cell line stimulated in step (b);

d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c);

30 e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor

-122-

CD8+CD28- T cells; and

5 f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the xenospecific human suppressor CD8+CD28- T cells.

10 6. The method of claim 5 wherein the xenospecific mammalian antigen presenting cells (APCs) are selected from pig or primate APCs.

15 7. The method of claim 5 wherein the xenogeneic MHC class I antigen is selected from the group consisting of swine histocompatibility leukocyte antigen (SLA) class-I and MHC class II antigen is selected from the group consisting of swine histocompatibility leukocyte antigen (SLA) class-II.

20 8. Xenospecific human suppressor CD8+ CD28+ T cells produced by the method of claim 5.

25 9. A method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells which comprises:

a) obtaining peripheral blood T cells from a subject;

30 b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with autologous antigen presenting cells (APCs) pulsed with an allopeptide, said allopeptide comprising an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences and are recognized by the primed T cell line;

35 c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b);

-123-

d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c);

5 e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and autologous antigen presenting cells (APCs) expressing the same MHC class I and MHC class II binding motifs as expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying allopeptide antigen specific human suppressor CD8+CD28- T cells; and

10 f) expanding the allopeptide antigen specific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the allopeptide antigen specific human suppressor CD8+CD28- T cells.

15 10. The method of claim 9 wherein the allopeptide is selected from the group consisting of a peptide antigen, a whole protein antigen, tat-DR4 peptide or a peptide comprising an amino acid sequence of a hypervariable region of HLA-DR B1.

20 25 11. Antigen specific human suppressor CD8+CD28- T cells produced by the method of claim 9.

30 12. A method of determining whether a level of immunosupresant therapy given to a subject undergoing the level immunosuppression therapy requires a reduction which comprises:

a) obtaining a blood sample from the subject; and
35 b) determining the presence of T suppressor cells present in the sample,

-124-

the presence of T suppressor cells indicating that the subject requires the reduction of immunosuppressant therapy.

13. The method of claim 12 wherein the T suppressor cells
5 are suppressor CD8+CD28- T cells.

14. A method of reducing the risk of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises:

10 a) obtaining a blood sample from the subject;

15 b) removing T suppressor cells from the blood sample;

15 c) expanding the T suppressor cells of step (b); and

20 d) reintroducing the expanded T suppressor cells of step (b) into the subject.

20 15. The method of claim 14 wherein the T suppressor cells are suppressor CD8+CD28- T cells.

25 16. A method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the method of claim 1, thereby preventing rejection of the tissue or organ transplant in the subject.

30 17. A method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the method of claim 9, thereby preventing rejection of the tissue or organ transplant in the subject.

35 18. A method of preventing rejection of an allograft in a subject which comprises:

a) obtaining a blood sample from the subject;

-125-

- b) removing T suppressor cells from the blood sample;
- c) expanding the T suppressor cells of step (b); and
- 5 d) reintroducing the expanded T suppressor cells of step (b) into the subject,

thereby preventing the rejection of the allograft in the subject.

10 18. A method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the method of claim 1 to the subject, thereby preventing rejection of the allograft in the subject.

15 19. A method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the method of claim 9 to the subject, thereby preventing rejection of the allograft in the subject.

20 20. A method of preventing rejection of a xenograft in a subject which comprises:

- a) obtaining a blood sample from the subject;

25 b) removing T suppressor cells from the blood sample;

- c) expanding the T suppressor cells of step (b); and

30 d) reintroducing the expanded T suppressor cells of step (b) into the subject,

thereby preventing the rejection of the xenograft in the subject.

35 21. The method of claim 20 wherein the T suppressor cells are suppressor CD8+CD28- T cells.

-126-

22. A method of preventing rejection of a xenograft in a subject which comprises administering the T suppressor cells produced by the method of claim 5 to the subject, thereby preventing rejection of the xenograft in the subject.

5

23. A method of preventing autoimmune disease in a subject which comprises:

10

- a) obtaining a blood sample from the subject;
- 15 b) removing T suppressor cells from the blood sample;
- c) expanding the T suppressor cells of step (b); and
- d) reintroducing the expanded T suppressor cells of step (b) into the subject,

thereby preventing autoimmune disease in the subject.

20

24. The method of claim 23 wherein the T suppressor cells are suppressor CD8+CD28- T cells.

25

25. A method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by the method of claim 1 to the subject, thereby preventing autoimmune disease in the subject.

30

26. A method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by the method of claim 9 to the subject, thereby preventing autoimmune disease in the subject.

35

27. A vaccine comprising allospecific T suppressor cells stimulated by APCs expressing an MHC class I antigen and an MHC class II antigen which T suppressor cells suppress an interaction between CD4+ T helper cells and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the

-127-

APCs used to stimulate the allospecific T suppressor cells.

28. The vaccine of claim 27 wherein the APCs are allogeneic
5 APCs said APCs expressing an MHC class I antigen recognized
by the T suppressor cells and an MHC class II antigen
recognized by allogeneic CD4+ T helper cells.

29. The vaccine of claim 27 wherein the APCs are APCs pulsed
10 with an allopeptide, said allopeptide comprising an amino
acid sequence having both MHC class I and MHC class II
binding motifs wherein both motifs are recognized by the
stimulated T suppressor cells.

30. The vaccine of claim 27 wherein the T suppressor cells
15 are suppressor CD8+CD28- T cells.

31. A vaccine comprising xenospecific T suppressor cells
20 stimulated by APCs expressing a xenospecific MHC class I
antigen and a xenogeneic MHC class II antigen which
xenogeneic T suppressor cells suppress an interaction between
CD4+ T helper cells and xenogeneic antigen presenting cells
(APCs) expressing the same xenogeneic MHC class I antigen and
xenogeneic MHC class II antigen expressed by the APCs used
to stimulate the xenospecific T suppressor cells.

25 32. The vaccine of claim 31 wherein the T suppressor cells
are suppressor CD8+CD28- T cells.

33. A method of inducing anergic T helper cells which
30 comprises:

- a) incubating antigen presenting cells (APC) with
allospecific T suppressor cells (Ts);
- b) overexpressing in the APC mRNA which encodes at least
one monocyte inhibitory receptor (MIR), in a mixture of
cells comprising the APCs from step (a), wherein
overexpression of MIR transmits negative inhibitory signals
35 to recruit an inhibitory signaling molecule, tyrosine

-128-

phosphatase SHP-1 such that the APC are rendered tolerogenic; and
c) incubating the APCs from step (b) with T helper cells (Th) to induce Th anergy.

5

34. The method of claim 33, wherein the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3.

10 35. The method of claim 33, wherein the Ts are allospecific human suppressor CD8+CD28- T cells.

15 36. The method of claim 33, wherein the Ts are xenospecific human suppressor CD8+CD28- T cells.

20 37. The method of claim 33, wherein the Ts allopeptide are antigen specific human suppressor CD8+CD28- T cells.

30 38. A method of generating a tolerogenic antigen presenting cell (APC) which comprises:

- a) contacting the APC with Ts; and
- b) overexpressing mRNA which encodes an MIR in the APC, thereby generating a tolerogenic antigen presenting cell (APC).

25

39. The method of claim 38, wherein the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3.

30

40. The method of claim 38, wherein the Ts are antigen specific allospecific human suppressor CD8+CD28- T cells.

41. The method of claim 38, wherein the Ts are xenospecific human suppressor CD8+CD28- T cells.

35

42. The method of claim 38, wherein the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

43. A method of reducing the level of rejection of an allograft tissue or organ in a subject who is a transplant recipient of the allograft tissue or organ which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), wherein the APC have been incubated with Ts prior to overexpression of MIR, thereby inducing Th anergy so as to prevent rejection of the tissue or organ allograft in the subject.

44. The method of claim 43, wherein the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3.

45. The method of claim 43, wherein the Ts are allospecific human suppressor CD8+CD28- T cells.

46. The method of claim 43, wherein the Ts are xenospecific human suppressor CD8+CD28- T cells.

47. The method of claim 43, wherein the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

48. A method of suppressing an autoimmune disease in a subject which comprises:

a) contacting antigen presenting cells (APC) of the subject with T suppressor cells (Ts) specific for the antigen which induces the autoimmune disease; and

b) administering to the subject the APC of step(a),

thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

49. The method of claim 48, wherein the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3.

-130-

50. The method of claim 48, wherein the Ts are allospecific human suppressor CD8+CD28- T cells.

5 51. The method of claim 48, wherein the Ts are xenospecific human suppressor CD8+CD28- T cells.

52. The method of claim 48, wherein the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

10 53. A method of suppressing an autoimmune disease in a subject which comprises:

a) overexpressing monocyte inhibitory receptor (MIR) in antigen presenting cells (APC) of the subject, which APC present the antigen which induces the autoimmune disease and are genetically engineered to overexpress MIR; and
b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

20 54. The method of claim 53, wherein the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3.

25 55. A method of inducing tolerance to an allograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the allograft in the subject.

30

35

-131-

56. A method of inducing tolerance to a xenograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the xenograft in the subject.

5

10

15

RECEIVED
PATENT AND TRADEMARK OFFICE
U.S. DEPARTMENT OF COMMERCE
JULY 2000

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
21 December 2000 (21.12.2000)

PCT

(10) International Publication Number
WO 00/76320 A1

(51) International Patent Classification⁷: A01N 63/00,
C12N 5/02, 5/06, 5/08

York, NY 10019 (US). LIU, Zhuoru [US/US]; Apartment 9C, 100 Haven Avenue, New York, NY 10032 (US). CHANG, Chih-Chao [US/US]; Apartment 228, 555 Central Park Avenue, Scarsdale, NY 10583 (US).

(21) International Application Number: PCT/US00/16594

(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).

(22) International Filing Date: 15 June 2000 (15.06.2000)

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/333,809 15 June 1999 (15.06.1999) US

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:

US 09/333,809 (CIP)
Filed on 15 June 1999 (15.06.1999)

Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(71) Applicant (*for all designated States except US*): THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): SUCIU-FOCA, Nicole [US/US]; Apartment 19A, 120 Central Park South, New York, NY 10019 (US). CORTENSINI, Raffaello [IT/US]; Apartment 19A, 120 Central Park South, New

WO 00/76320 A1

(54) Title: GENERATION OF ANTIGEN SPECIFIC T SUPPRESSOR CELLS FOR TREATMENT OF REJECTION

(57) Abstract: This invention provides a method of generating antigen specific allospecific human suppressor CD8+CD28- T cells. This invention also provides a method of generating xenospecific human suppressor CD8+CD28- T cells. This invention further provides a method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells. Methods of treatment for reduction of risk of rejection of allografts and xenografts and autoimmune diseases using the human suppressor CD8+CD28- T cells so produced are also provided, as are methods of preventing rejection and autoimmune diseases, and vaccines comprising the produced suppressor T cells. Methods of diagnosis to determine whether a level of immuno-suppressant therapy requires a reduction are provided.

Applicant or Patentee: Nicole Suciu-Foca Attorney's
Serial or Patent No.: Not Yet Known Docket No: 58332/JPW/EMW
Filed or Issued: Herewith
Title of Invention or Patent: GENERATION OF ANTIGEN SPECIFIC T SUPPRESSOR CELLS
FOR TREATMENT OF REJECTION

VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The Trustees of Columbia University in the City of New York

Address of Organization: West 116th Street and Broadway
New York, New York 10027

TYPE OF ORGANIZATION:

UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and
501(c)(3)

NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED
STATES OF AMERICA

NAME OF STATE: _____

CITATION OF STATUTE: _____

WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C.
§§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA

WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE
OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

NAME OF STATE: _____

CITATION OF STATUTE: _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled GENERATION OF ANTIGEN SPECIFIC T SUPPRESSOR CELLS FOR TREATMENT OF REJECTION

by inventor(s) Nicole Suciu-Foca

described in:

the specification filed herewith
application serial no. _____ filed _____
patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

^aNOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

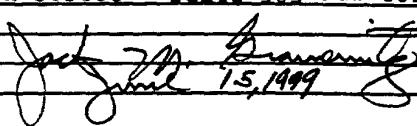
Name: N/A

Address: _____

Individual Small Business Concern Nonprofit Organization

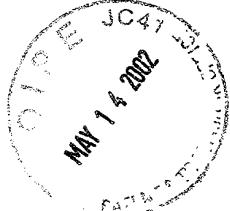
I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Mr. Jack M. Granowitz
Title In Organization: Executive Director, Columbia Innovation Enterprise
Address: Amsterdam & 120th Street - Suite 363 New York, New York 10027
Signature: 
Date Of Signature: June 15, 1999

Applicant or Pattee: Nicole Suciu-Foca, et al.
Serial or Patent No.: 10/018,677
Filed or Issued: December 14, 2001
Title of Invention or Patent: Generation of Antigen Specific T Suppressor Cells
For Treatment of Rejection

Attorney's
Docket No: 58332-A-PCT-US



VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The TRustees of Columbia University In the City of New York

Address of Organization: West 116th Street and Broadway
New York, New York 10027

TYPE OF ORGANIZATION:

UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
 TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3)
 NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA

NAME OF STATE:

CITATION OF STATUTE:

WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA

WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

NAME OF STATE:

CITATION OF STATUTE:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled Generation of Antigen Specific T Suppressor Cells For Traetment of Rejection

by inventor(s) Nicole Sucui-Foca, et al.

described in:

the specification filed herewith
 application serial no. 10/018,677 filed December 14, 2001
 patent no. issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below^a and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

^aNOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: N/A

Address:

Small Entity/Nonprofit
Page -2-

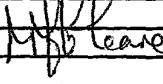
I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Mr. Michael J. Cleare

Title In Organization: Executive Director, Columbia Innovation Enterprise

Address: Amsterdam and 120th Street - Suite 363, New York, New York 10027

Signature: 

Date Of Signature: 1/2/02

1/44

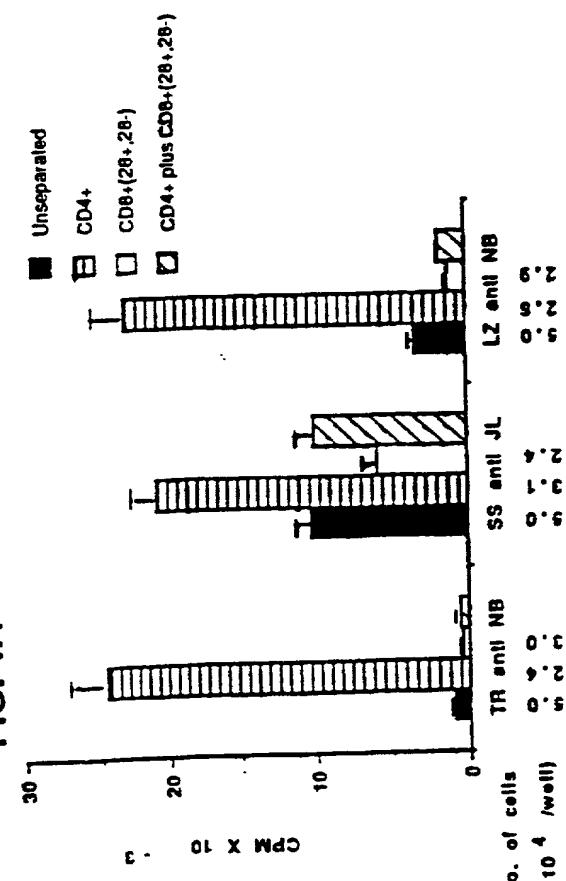
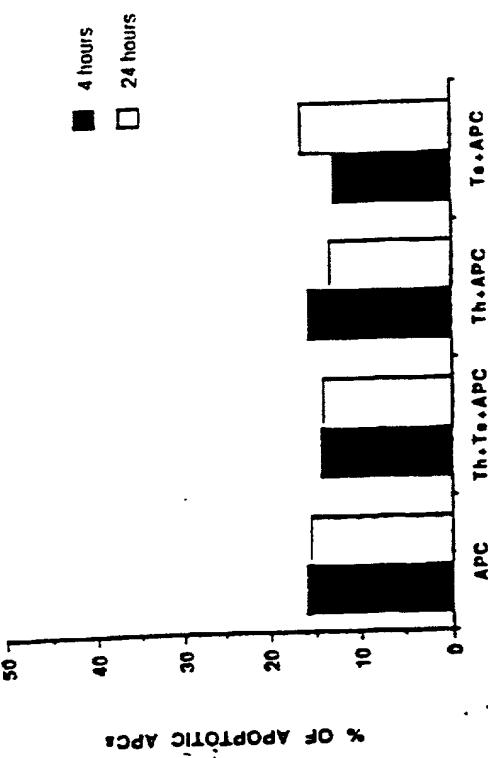
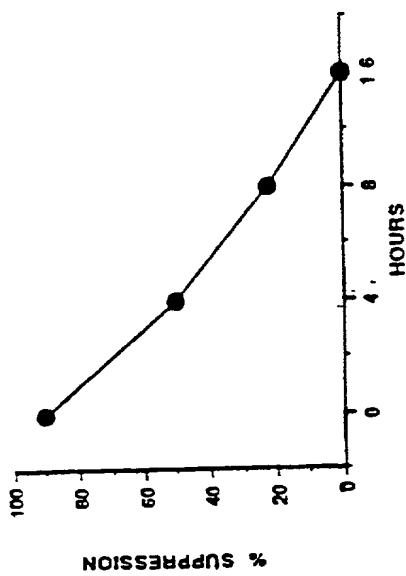
FIG. 1A**FIG. 1C****FIG. 1B****FIG. 1D**

FIG. 2A

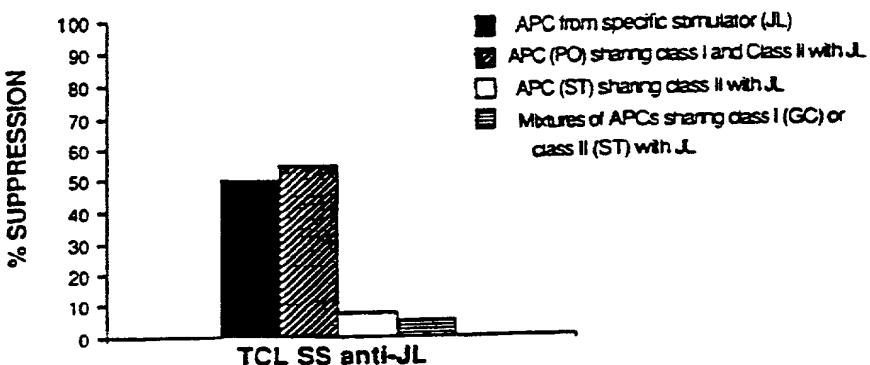
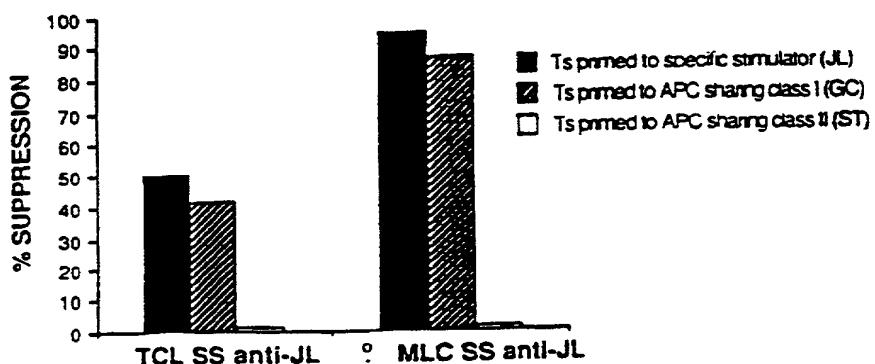
A

FIG. 2B

B

3/44

FIG. 3

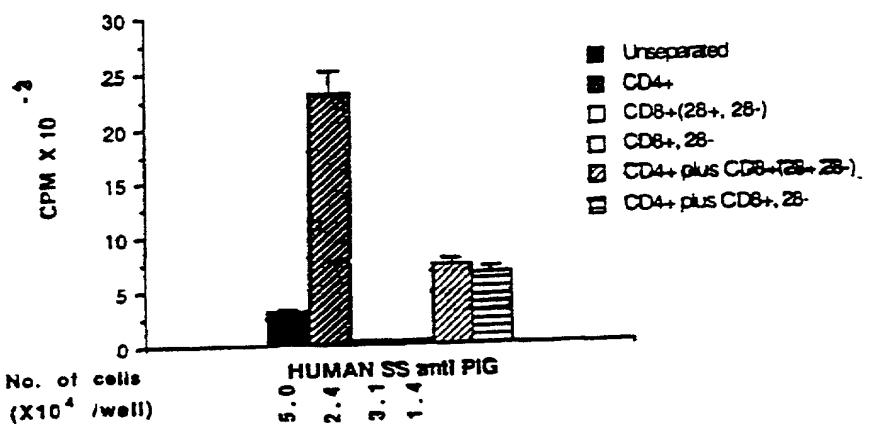
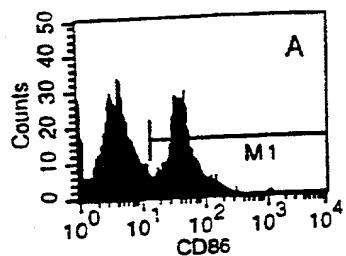
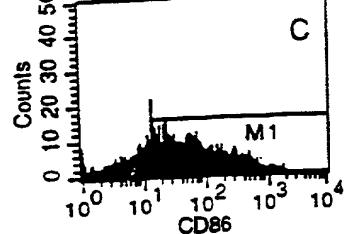
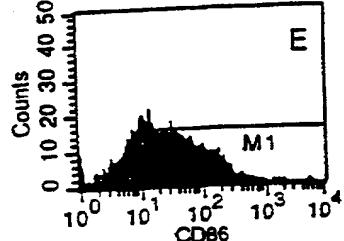
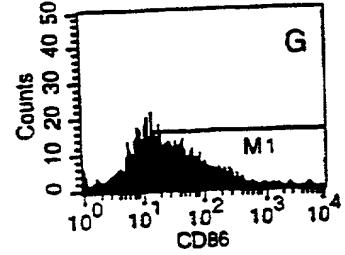


FIG. 4A

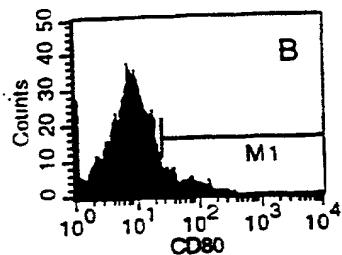
40 47

FIG. 4C

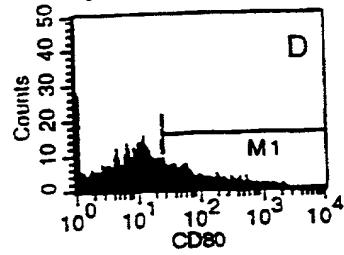
73 123

FIG. 4E**FIG. 4G**

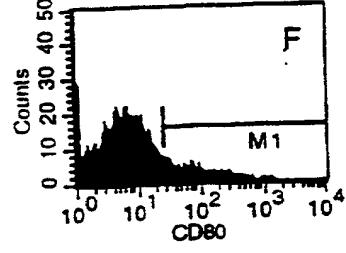
61 74

FIG. 4B

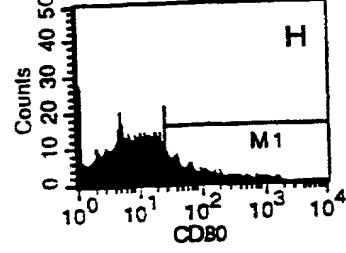
8 59

FIG. 4D

27 144

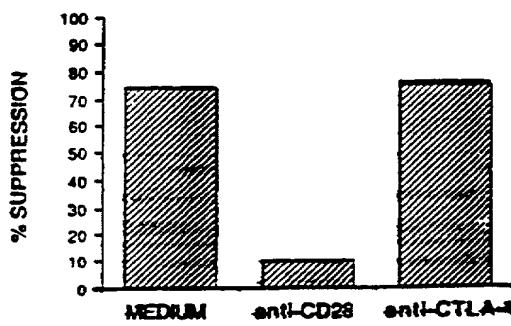
FIG. 4F

13 128

FIG. 4H

17 110

5/44

FIG. 5

6/44

FIG. 6A

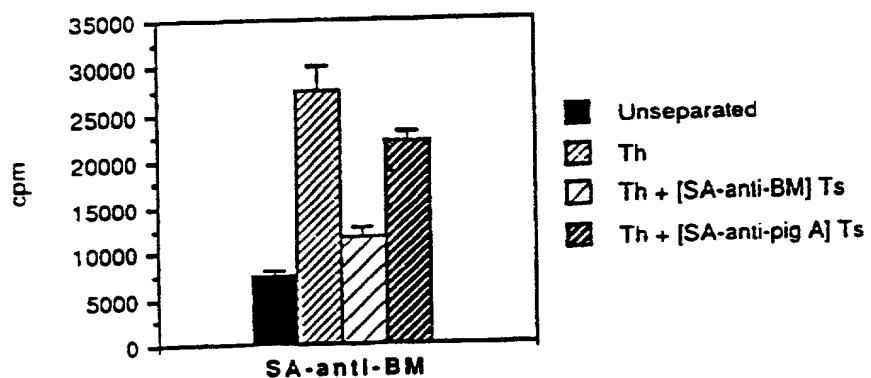


FIG. 6B

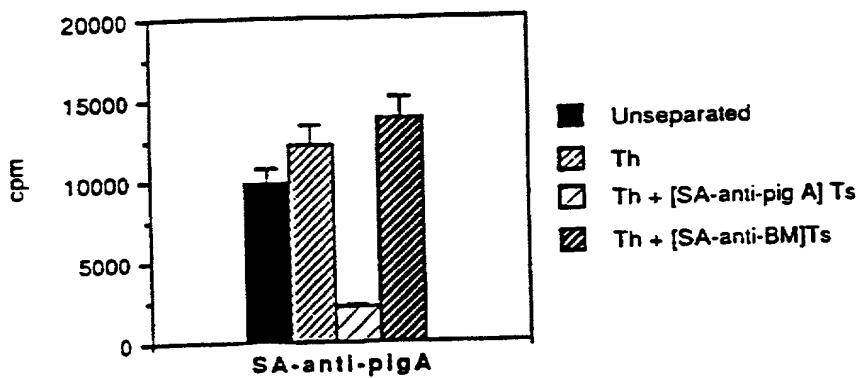
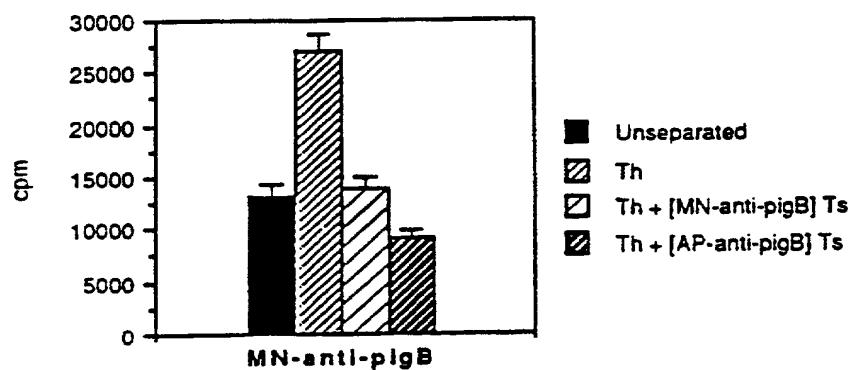
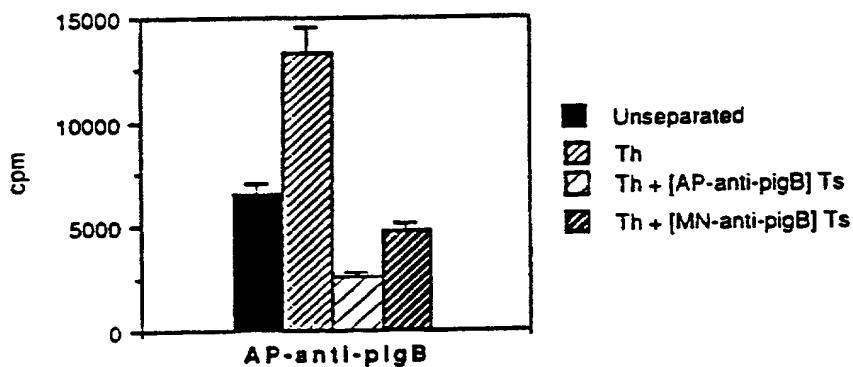
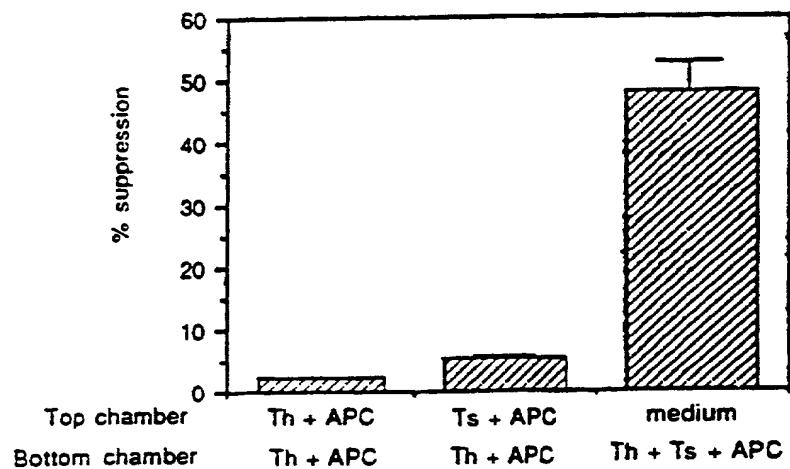
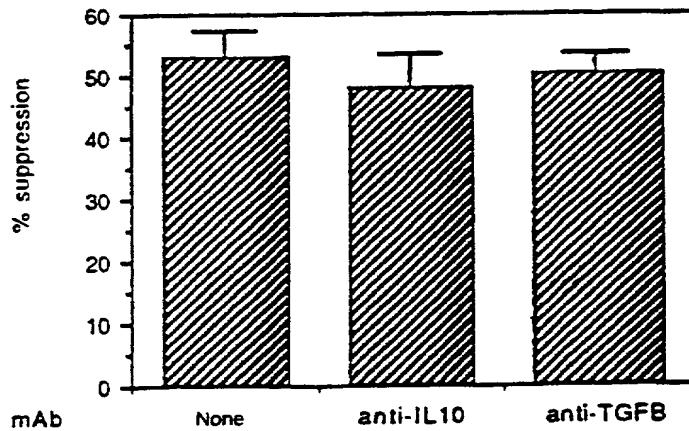


FIG. 7A**FIG. 7B**

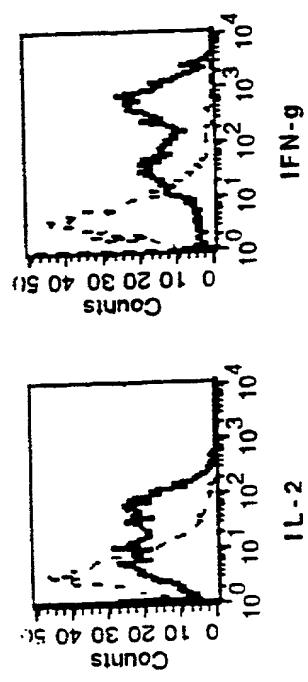
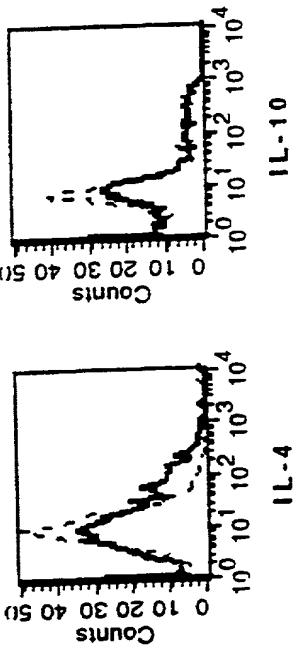
8/44

FIG. 8A**FIG. 8B**

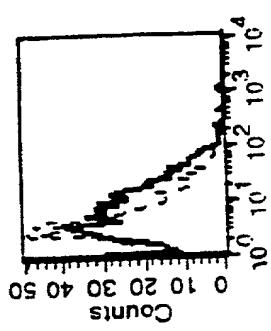
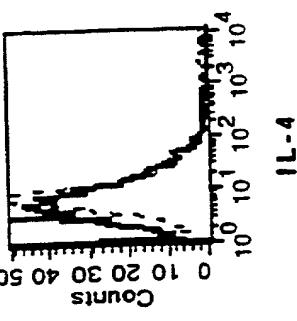
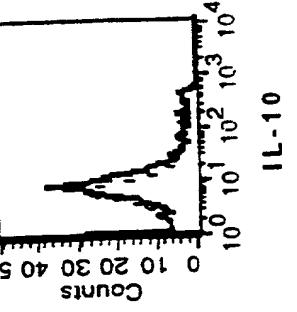
9/44

FIG. 9A

T helper cells

**FIG. 9C****FIG. 9D**

T suppressor cells

**FIG. 9E****FIG. 9F****FIG. 9G****FIG. 9H**

10/44

FIG. 10A

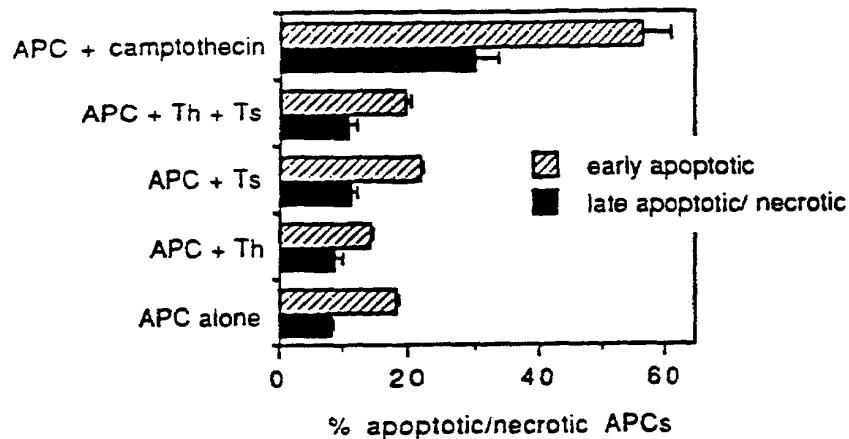


FIG. 10B

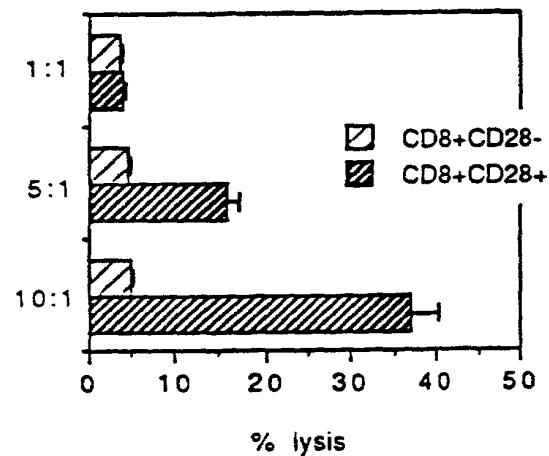
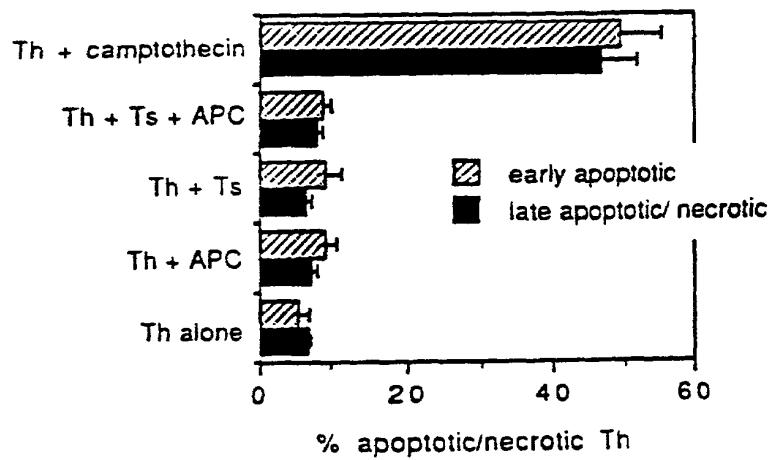


FIG. 10C



11/44

FIG. 11A

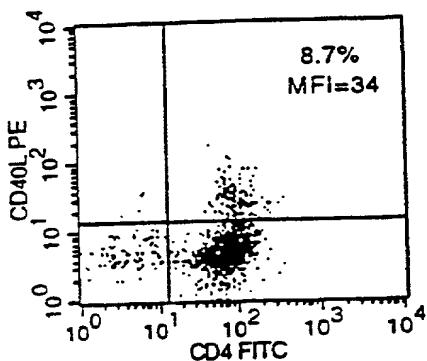


FIG. 11B

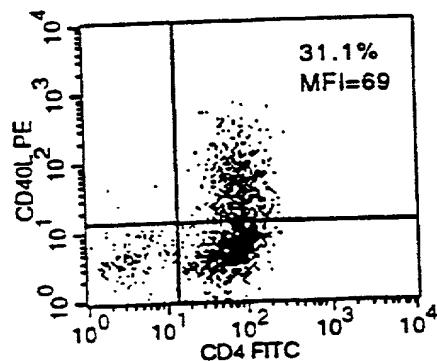


FIG. 11C

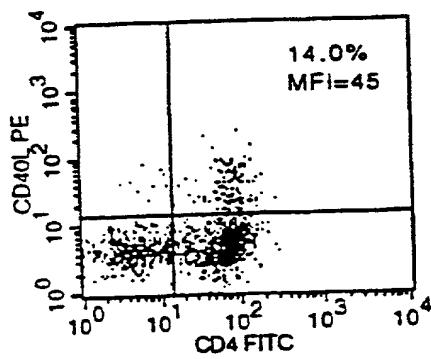
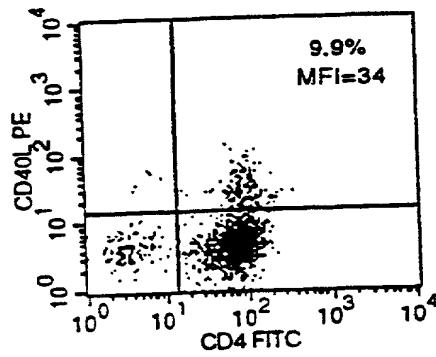
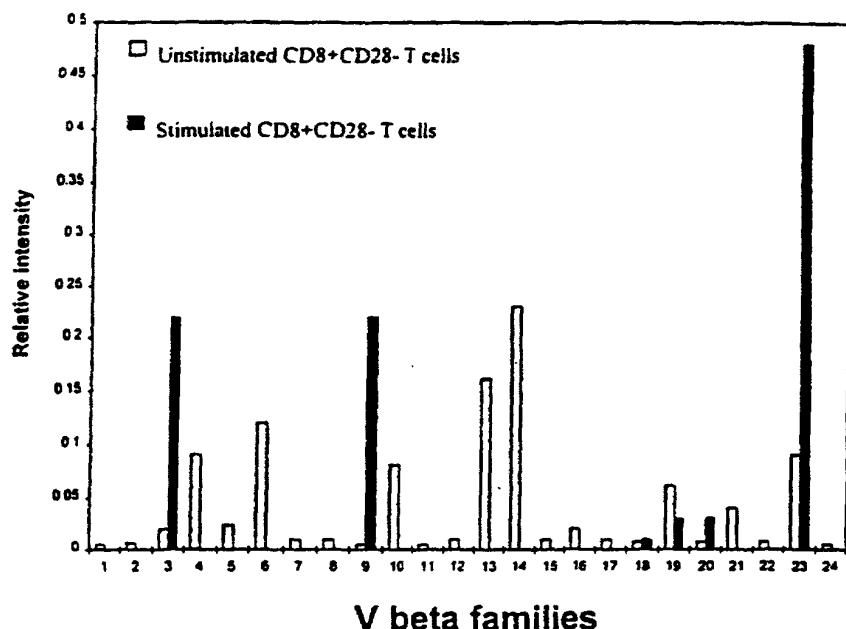


FIG. 11D



12/44

FIG. 12



13/44

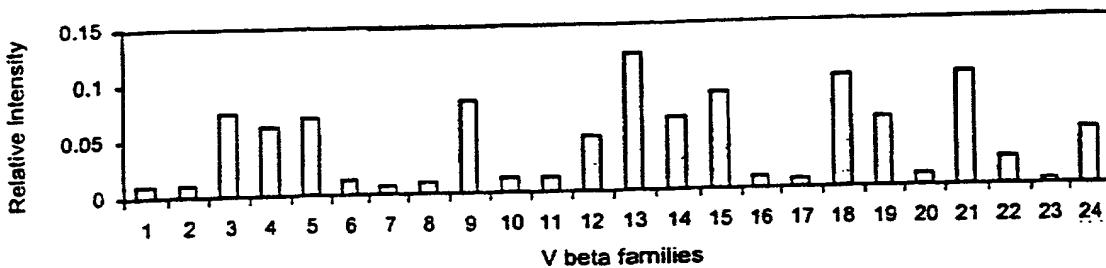
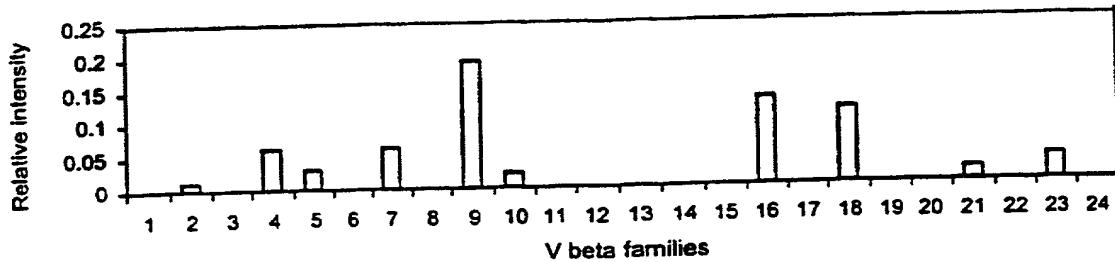
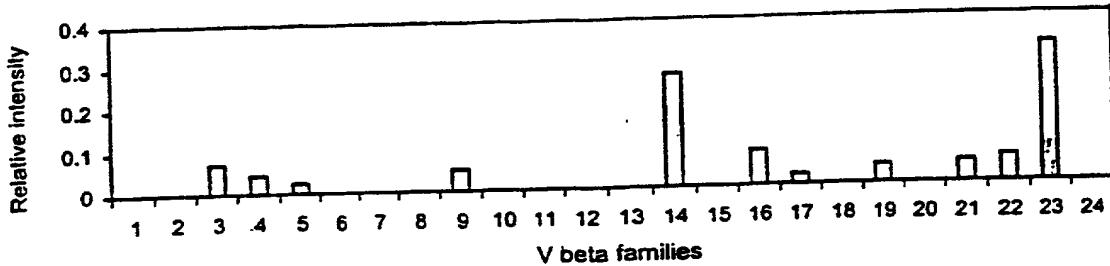
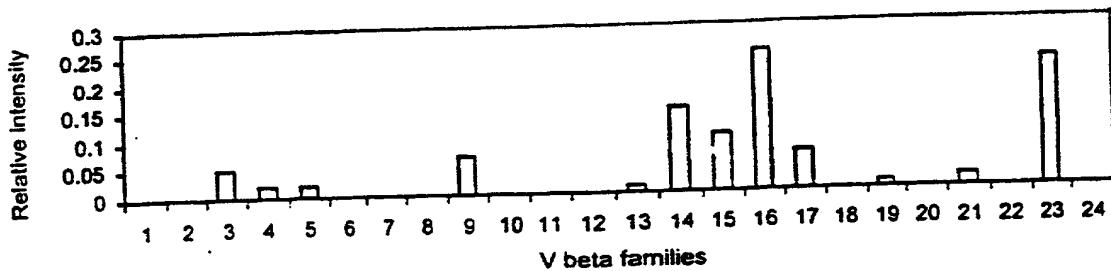
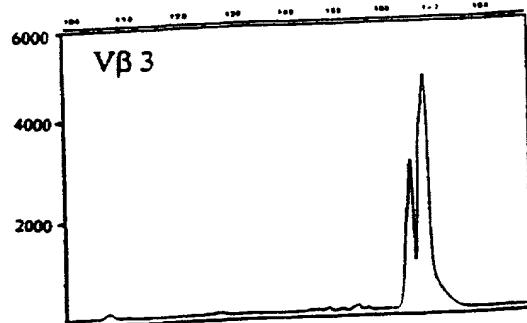
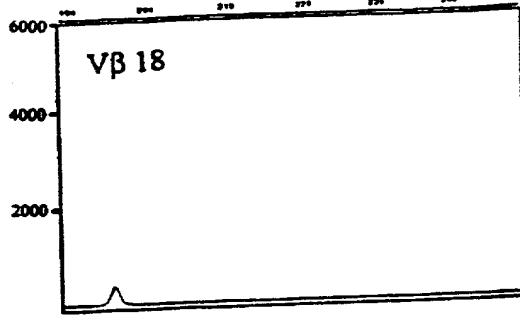
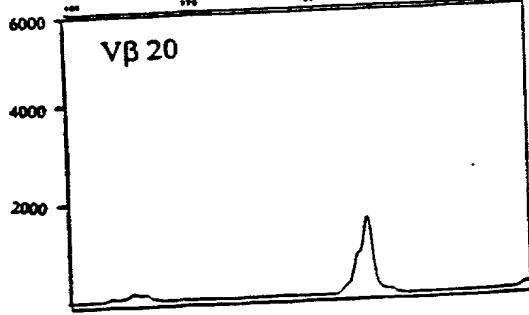
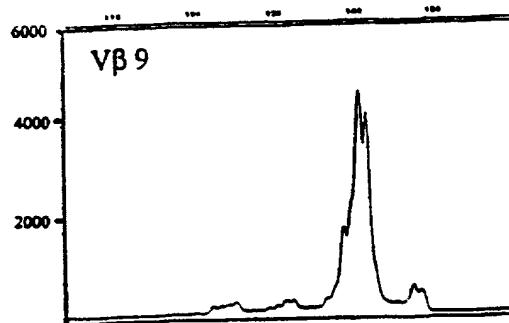
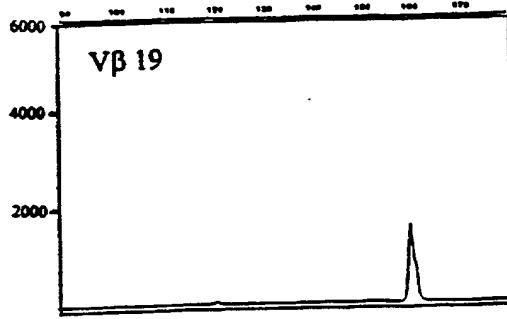
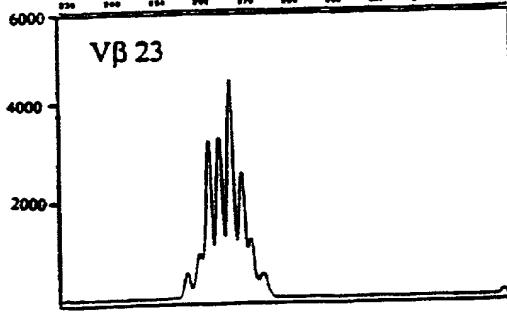
FIG. 13A**FIG. 13B****FIG. 13C****FIG. 13D**

FIG. 14A**FIG. 14D****FIG. 14E****FIG. 14B****FIG. 14C****FIG. 14F**

15/44

FIG. 15A

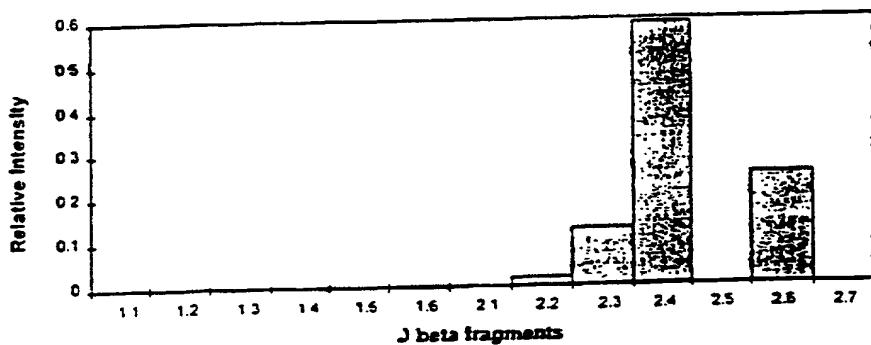


FIG. 15B

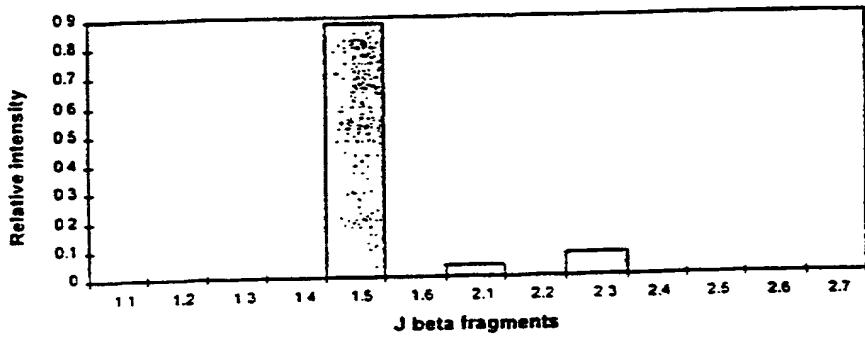
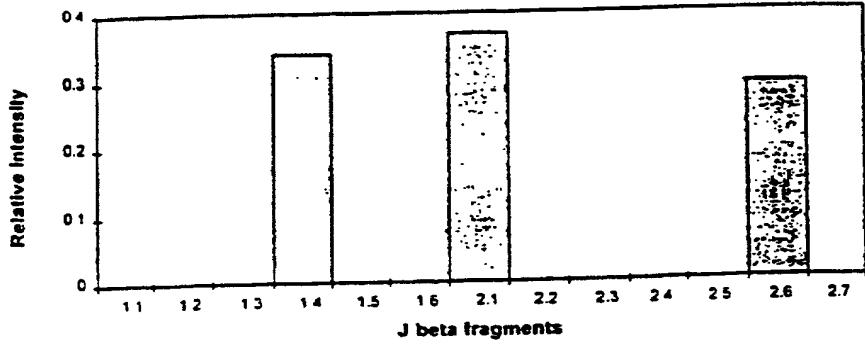
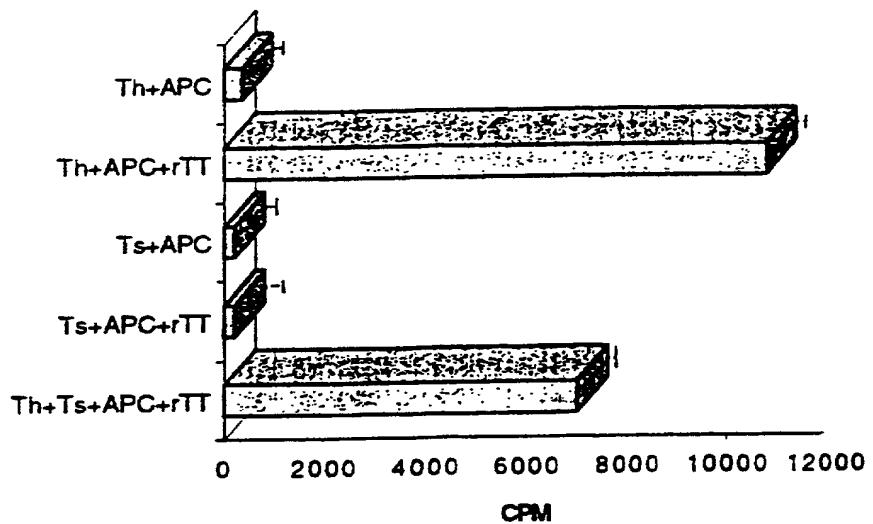


FIG. 15C



16/44

FIG. 16



10/018677

WO 00/76320

PCT/US00/16594

17/44

FIG. 17

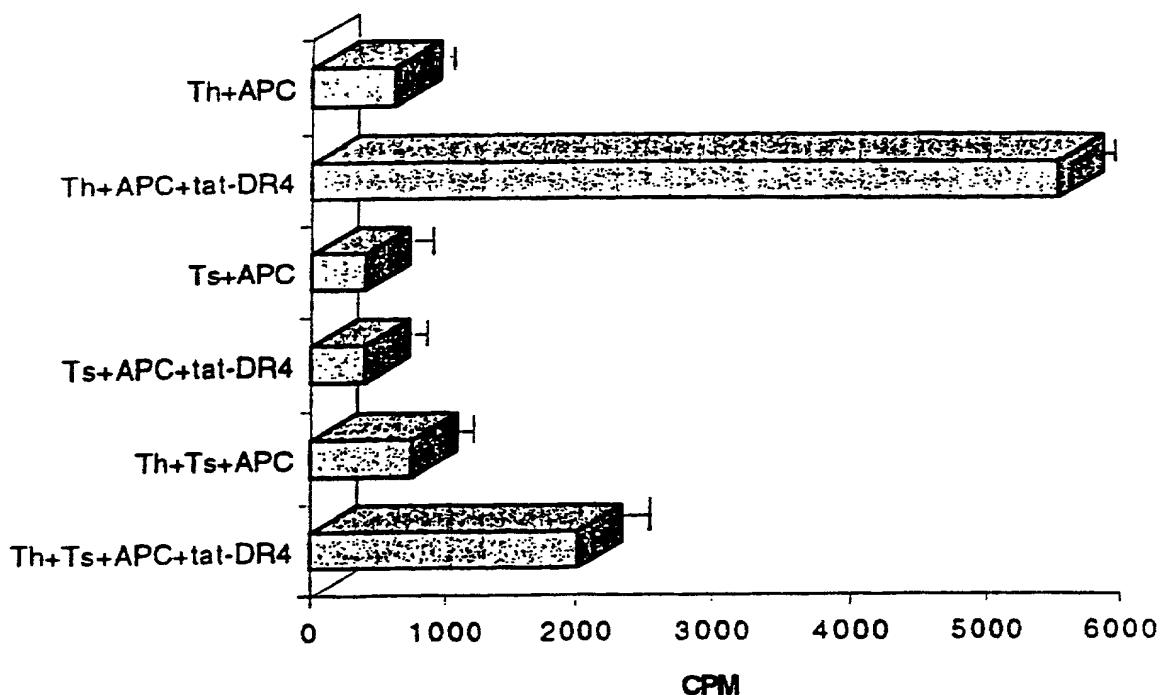


FIG. 18A

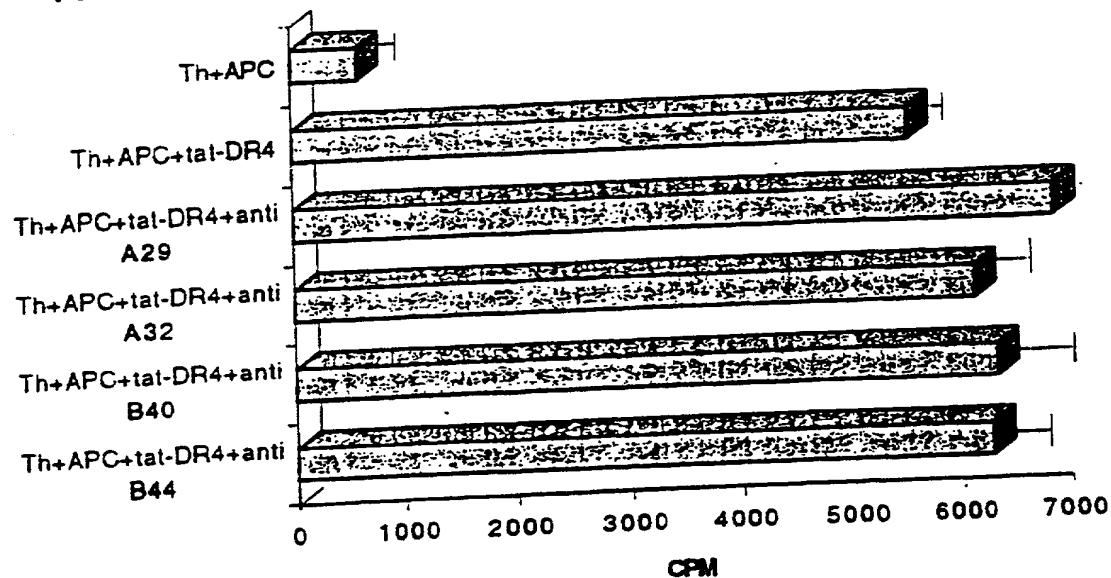
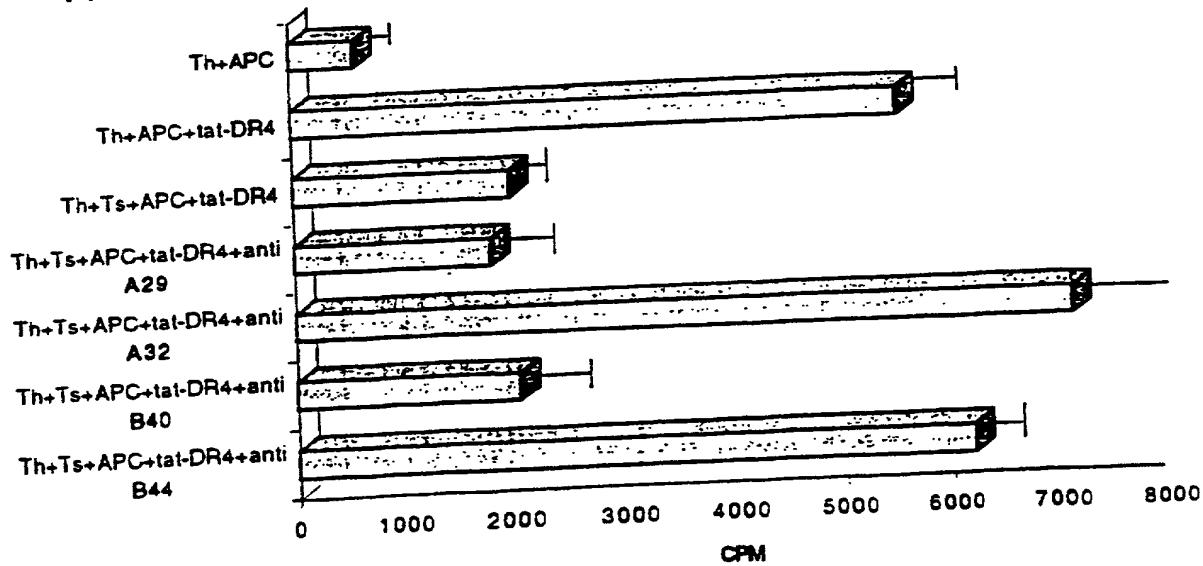


FIG. 18B

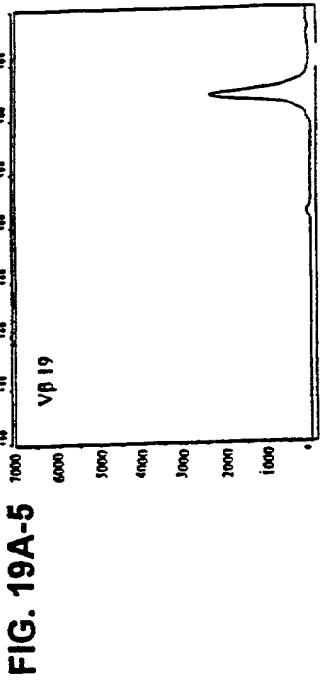
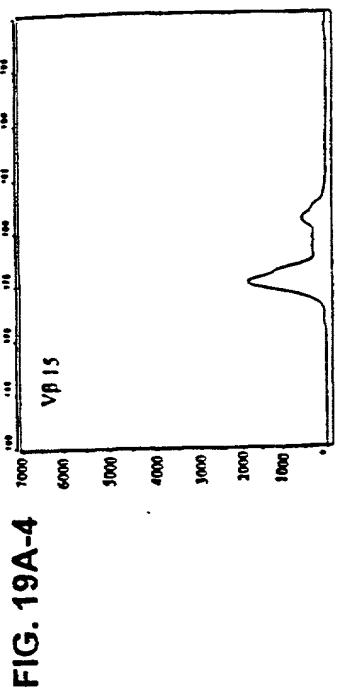
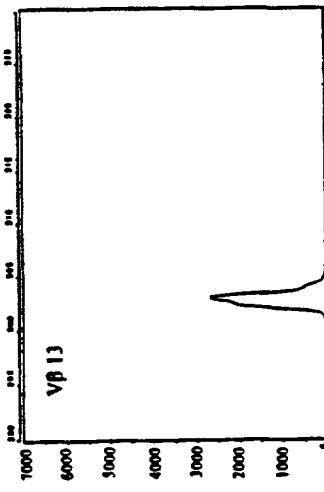
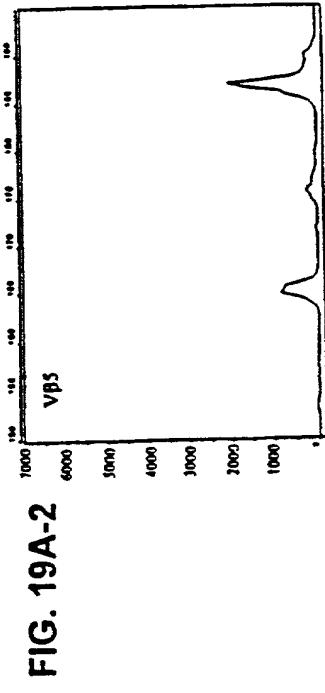
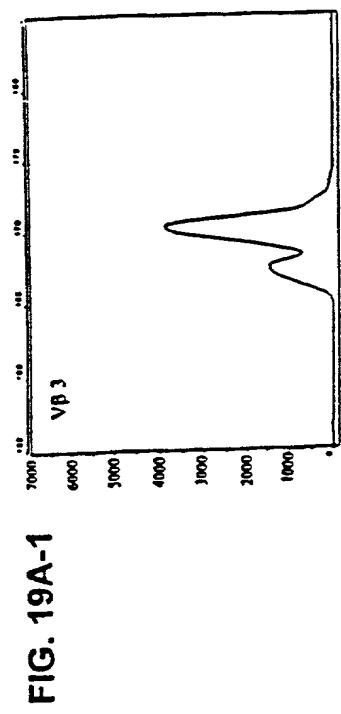


10/018677

WO 00/76320

PCT/US00/16594

19/44



100 200 300 400 500 600 700 800 900 1000

20/44

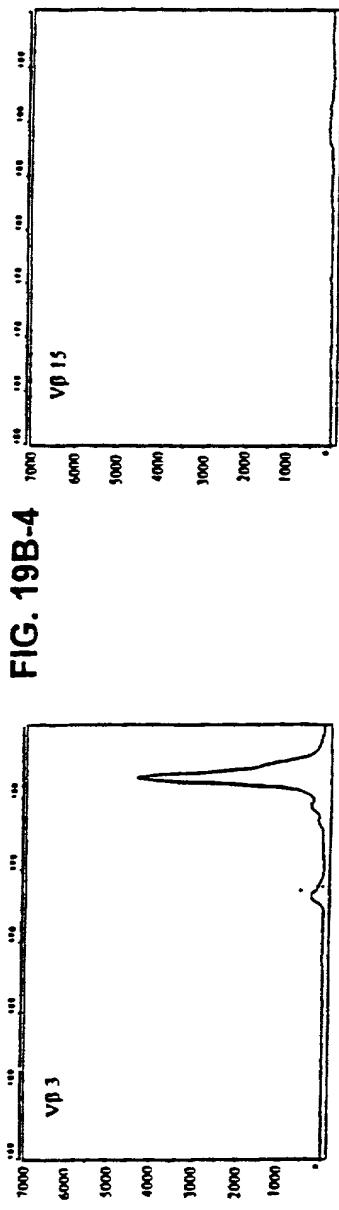
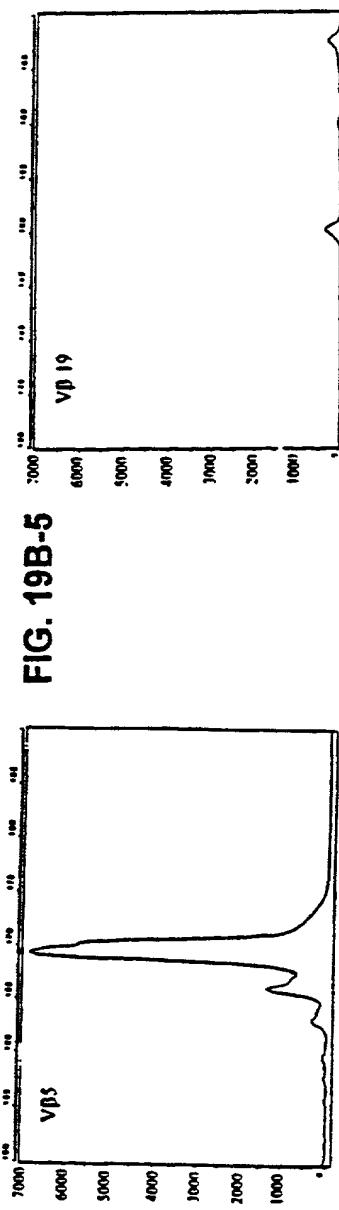
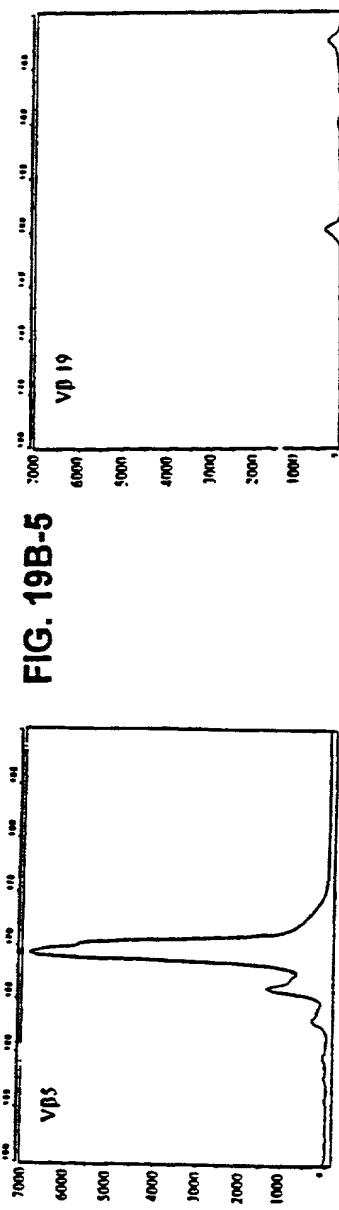
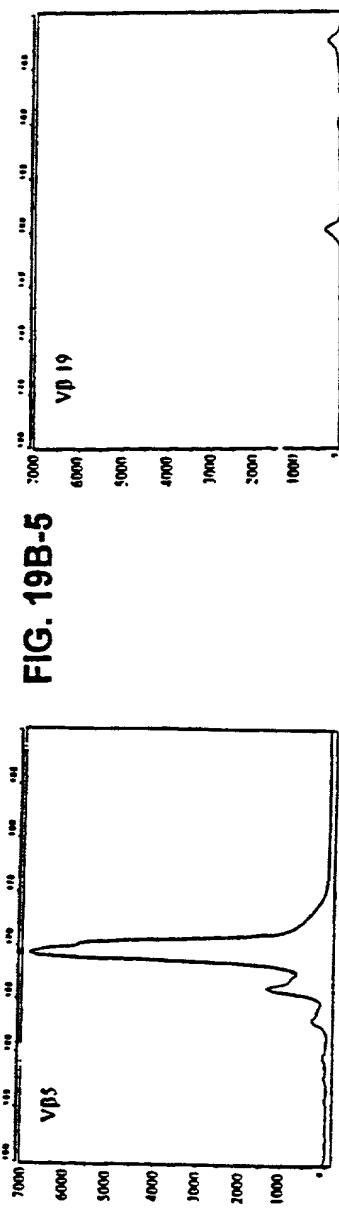
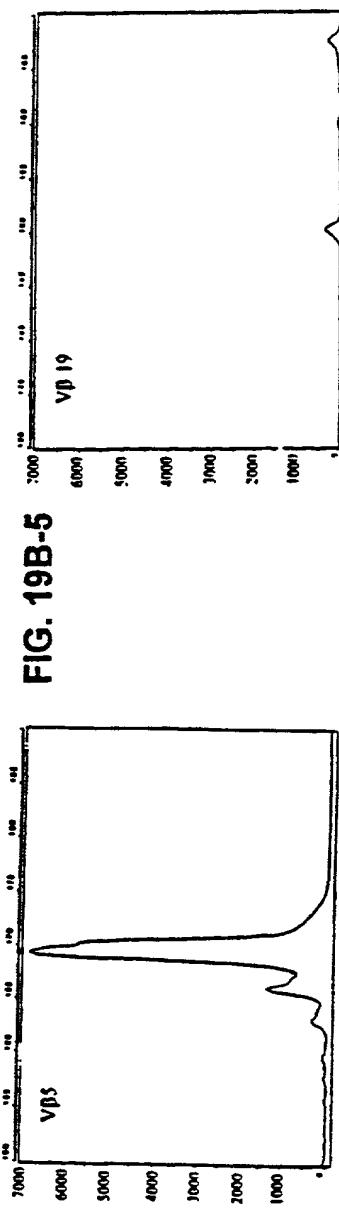
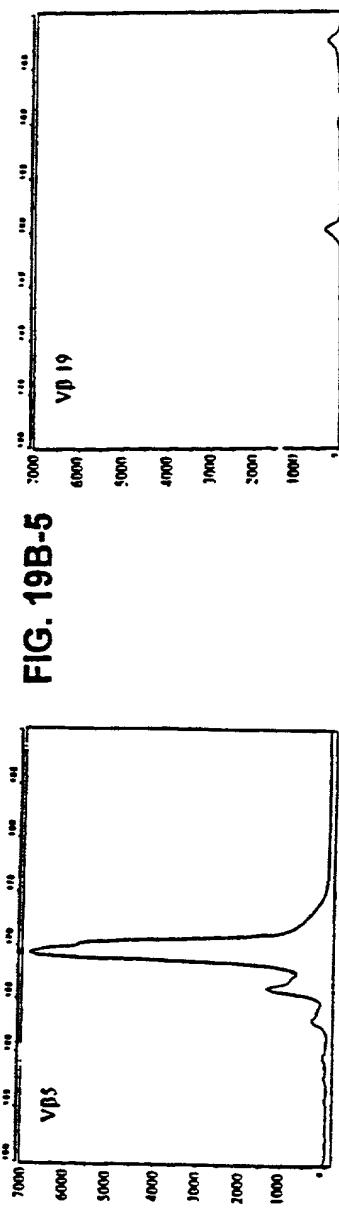
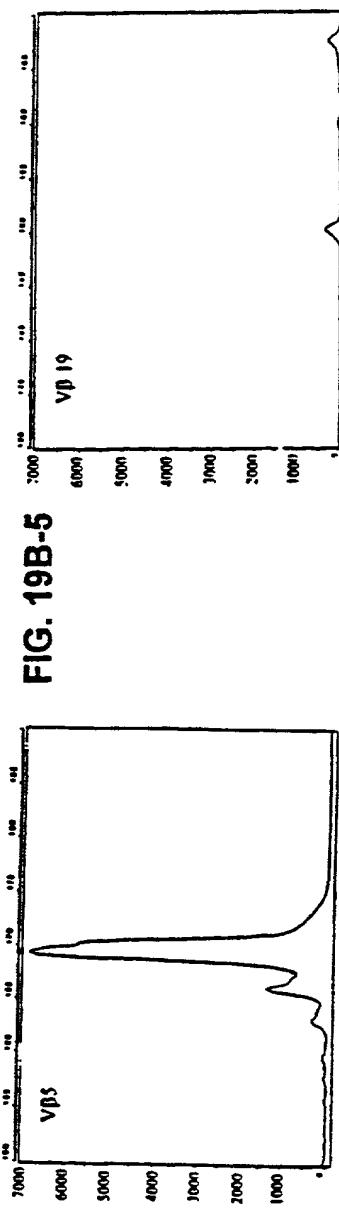
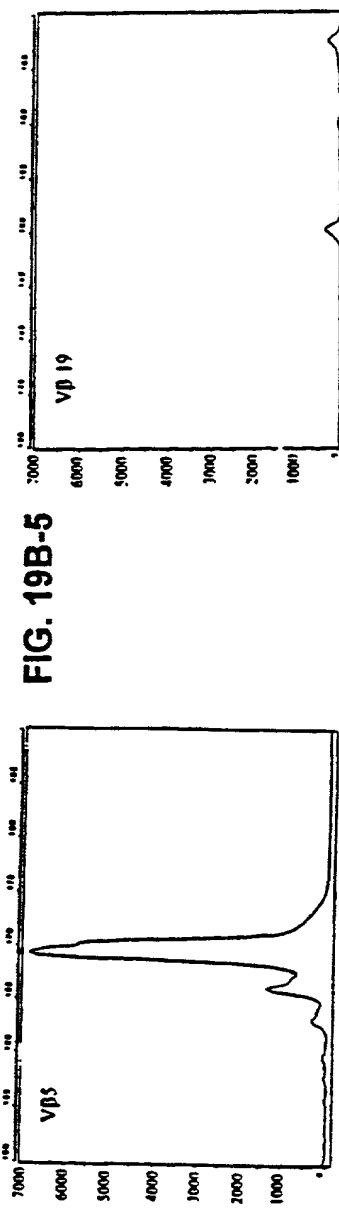
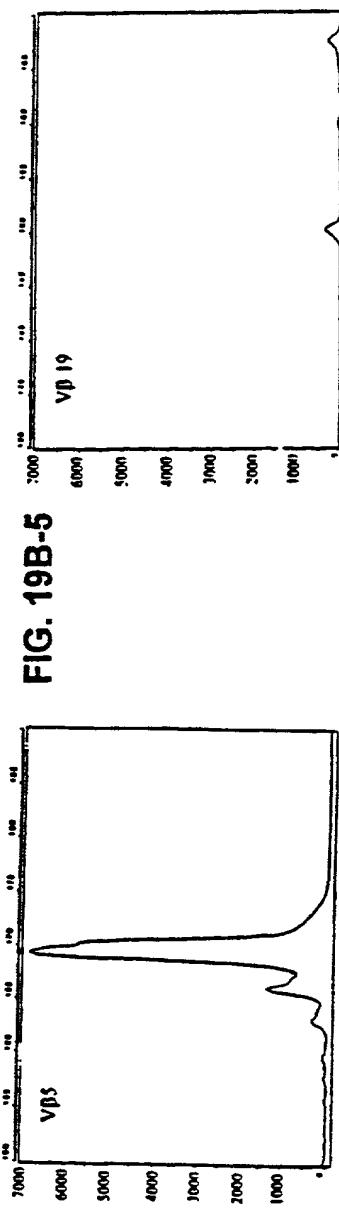
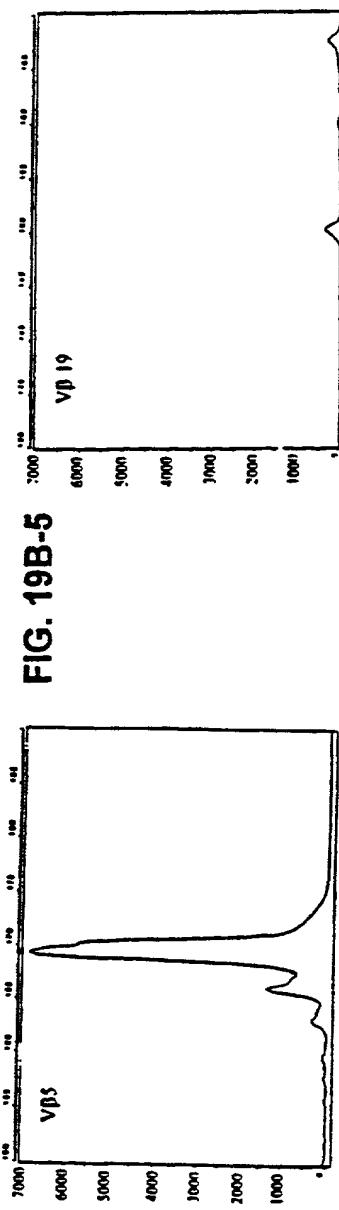
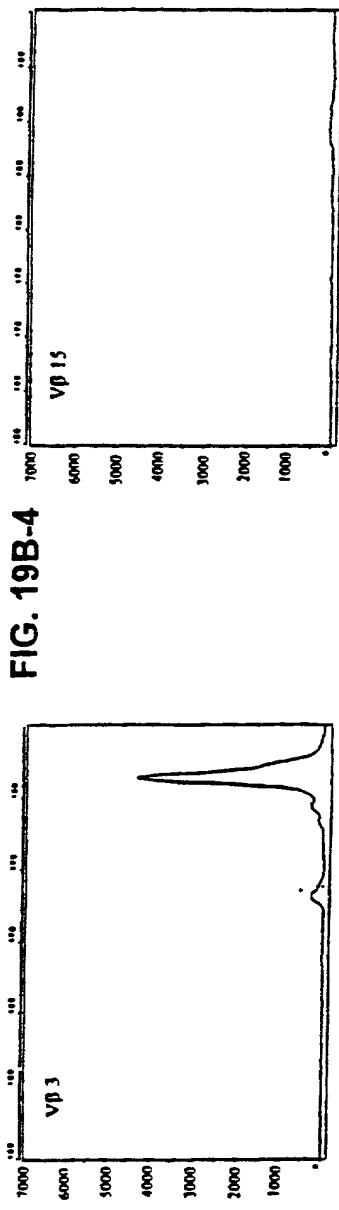
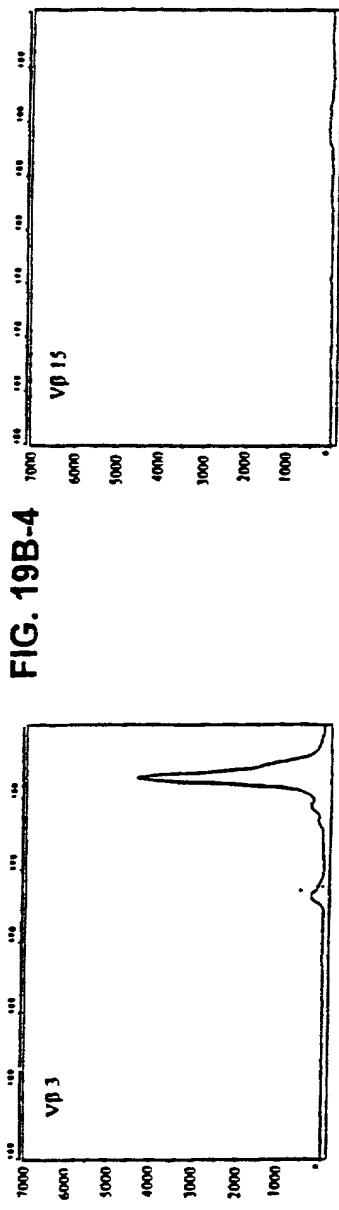
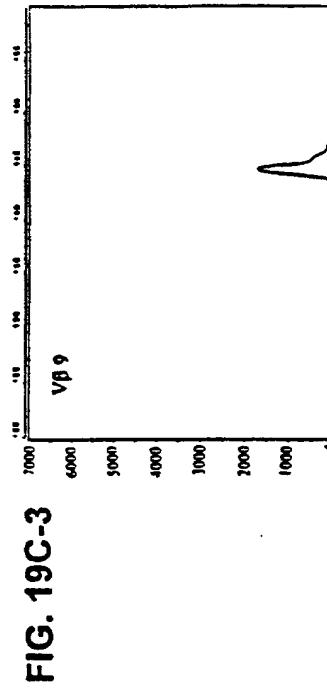
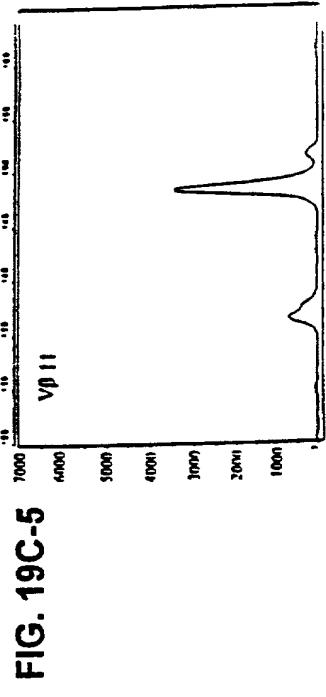
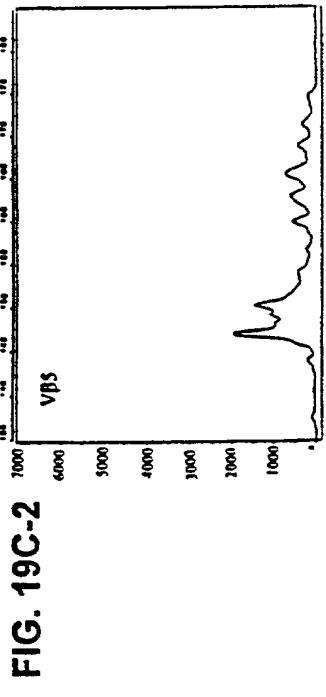
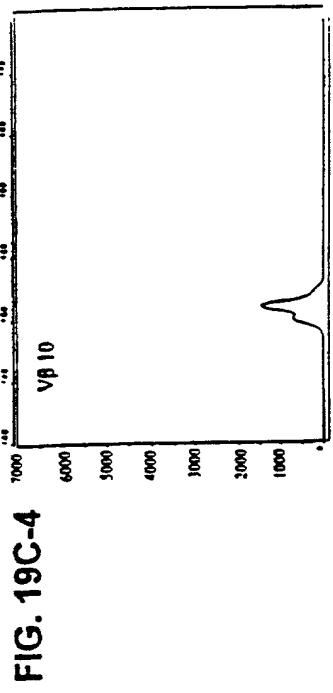
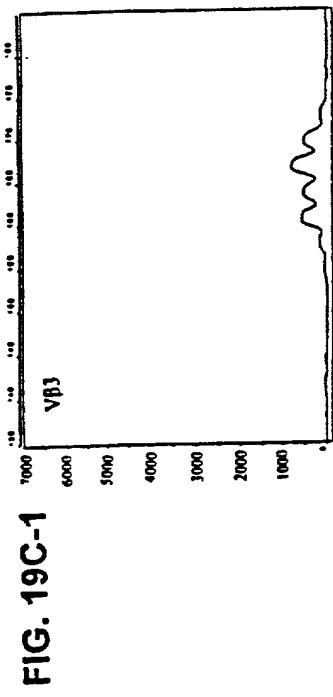
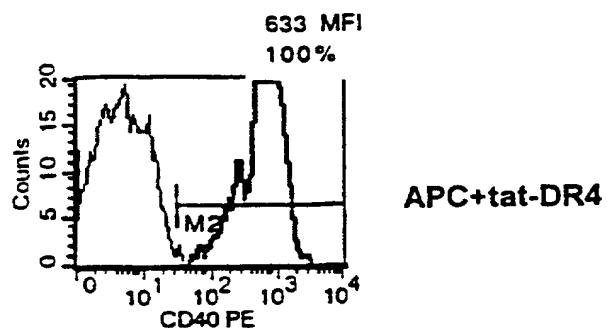
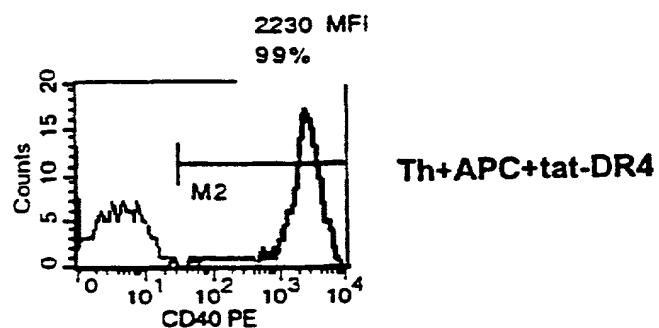
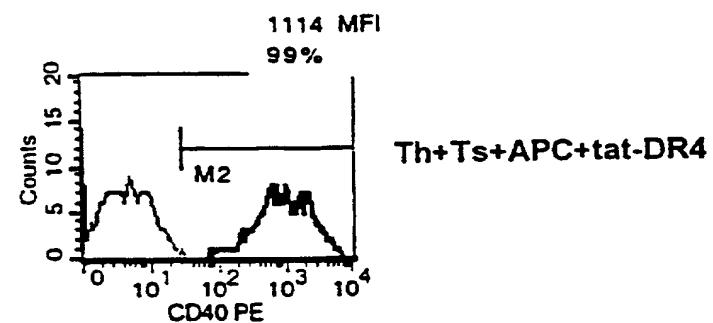
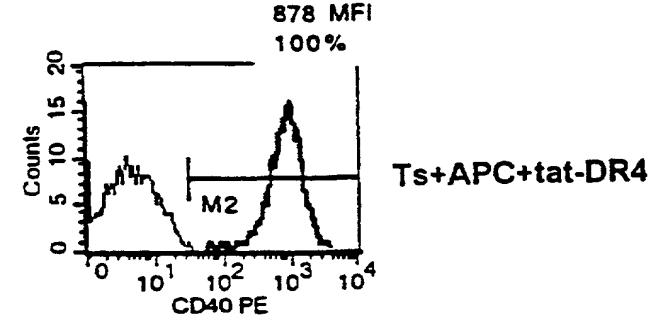


FIG. 19B-1

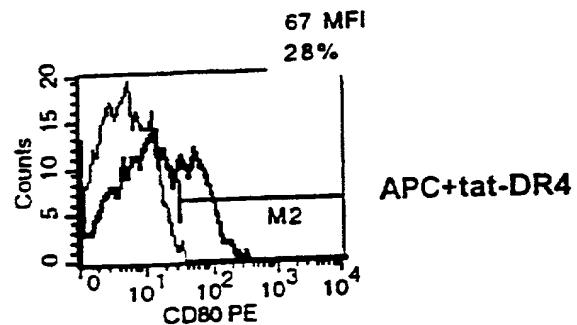
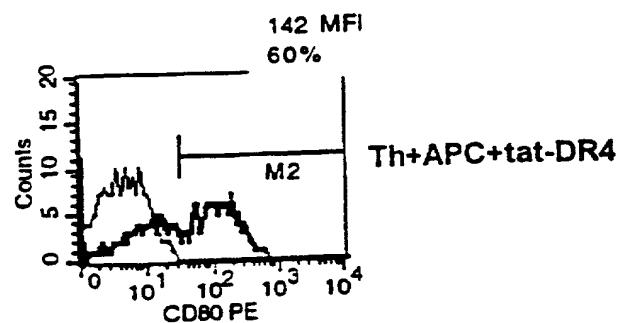
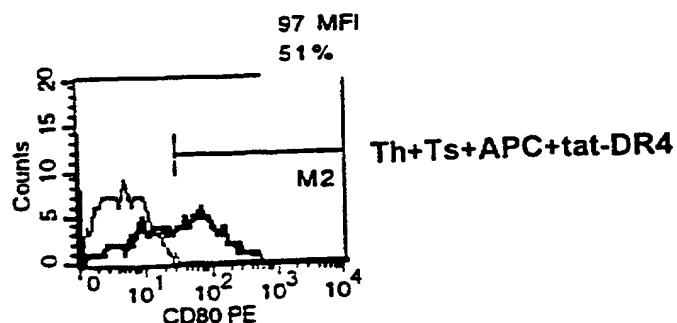
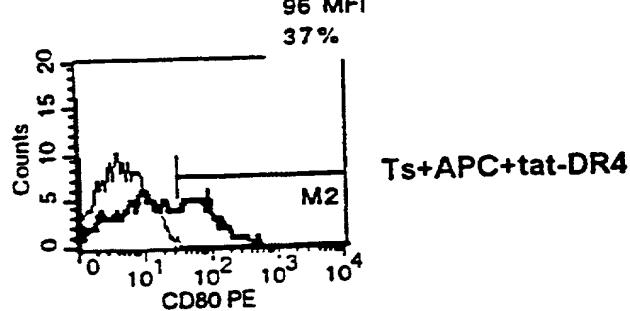




22/44

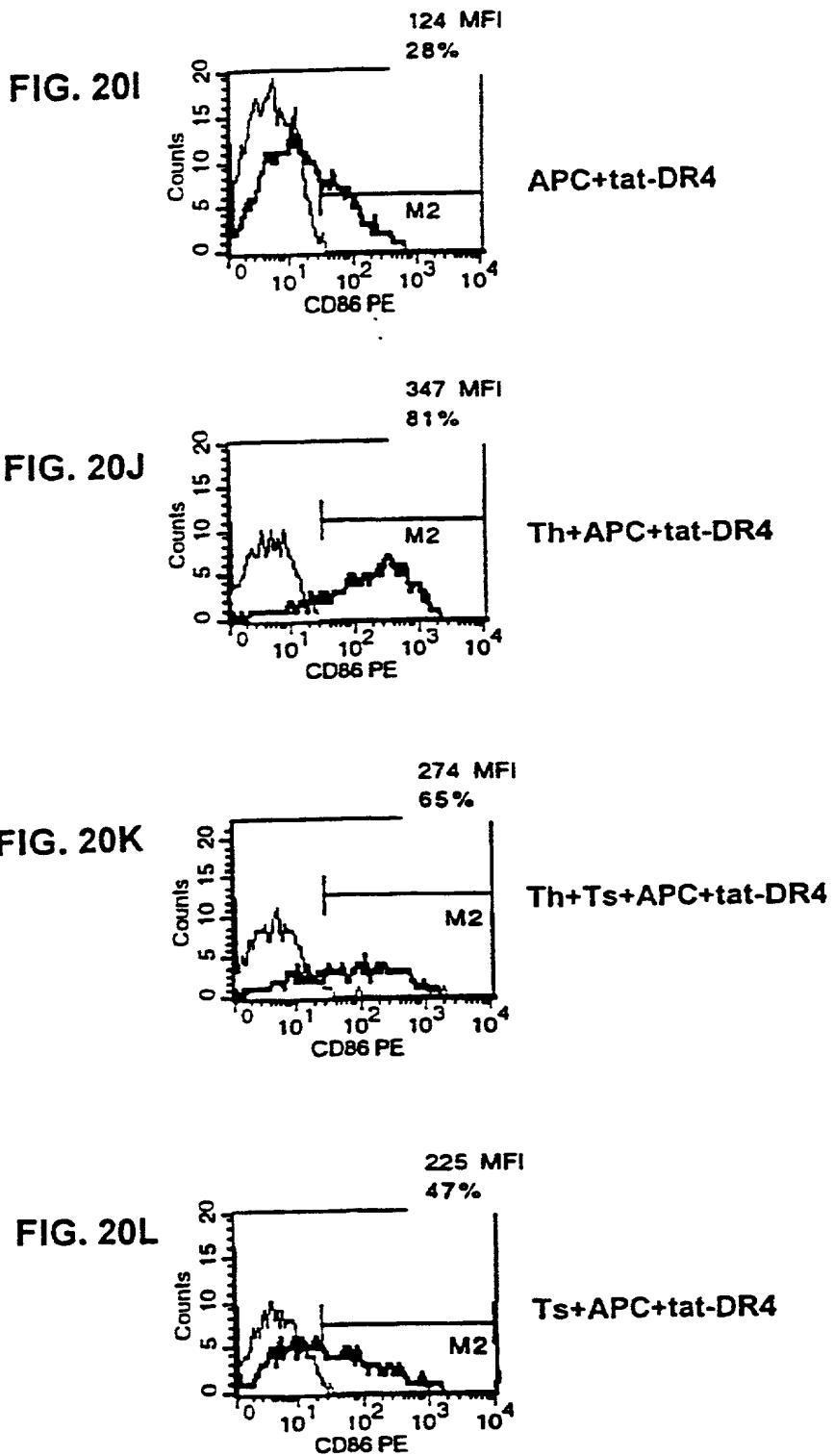
CD40**FIG. 20A****FIG. 20B****FIG. 20C****FIG. 20D**

23/44

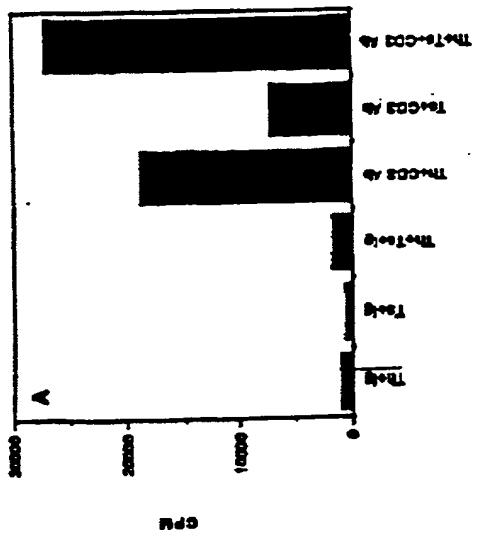
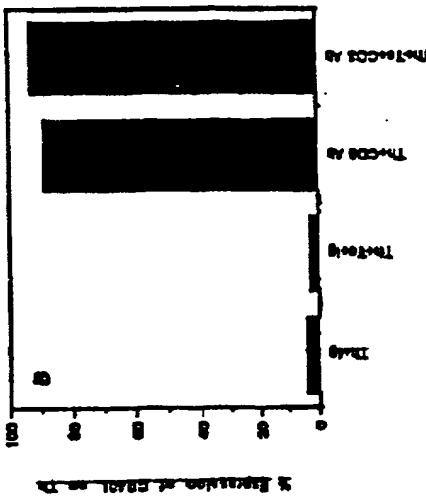
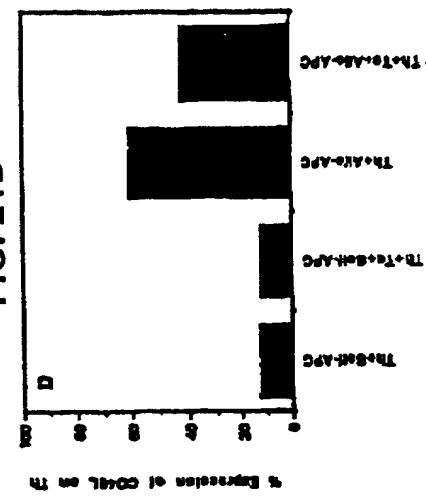
CD80**FIG. 20E****FIG. 20F****FIG. 20G****FIG. 20H**

24/44

CD86



25/44

FIG. 21A**FIG. 21B****FIG. 21C****FIG. 21D**

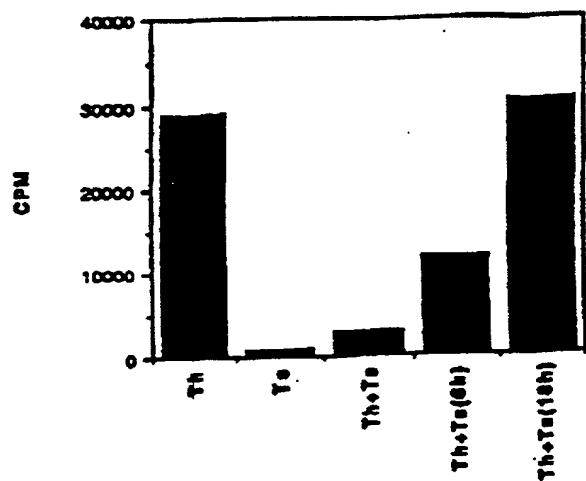
10/018677

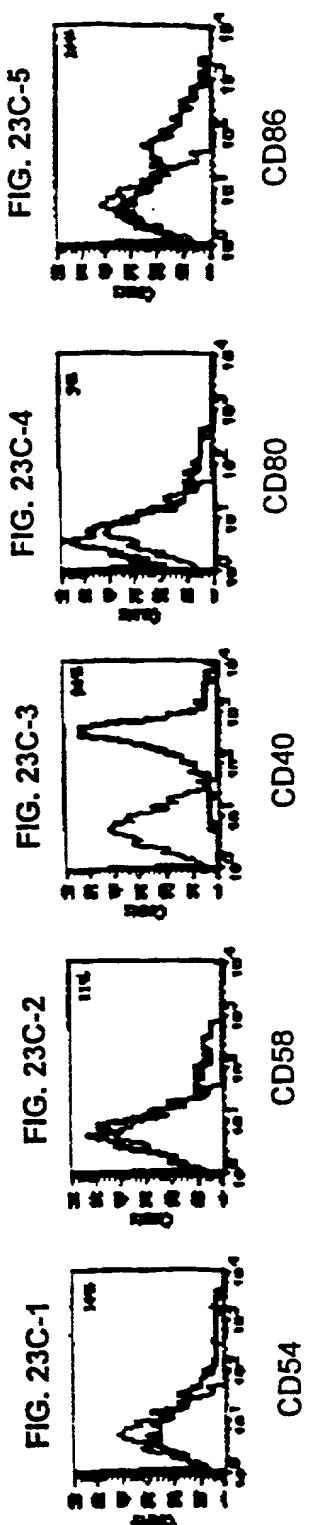
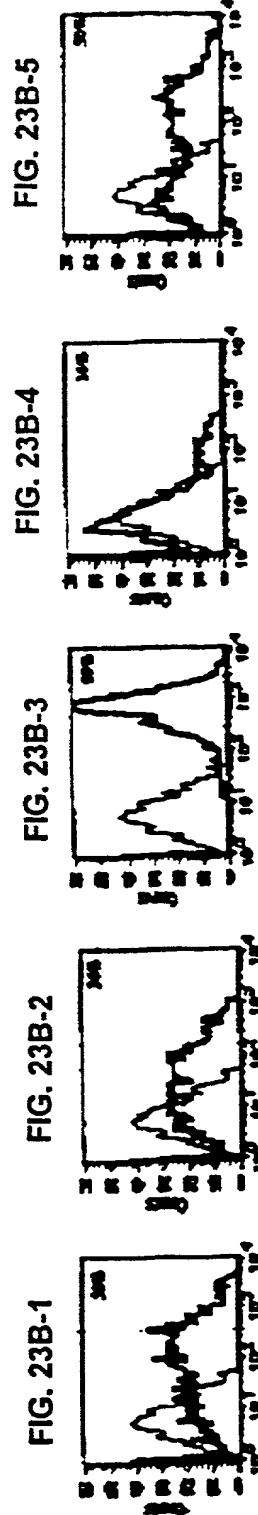
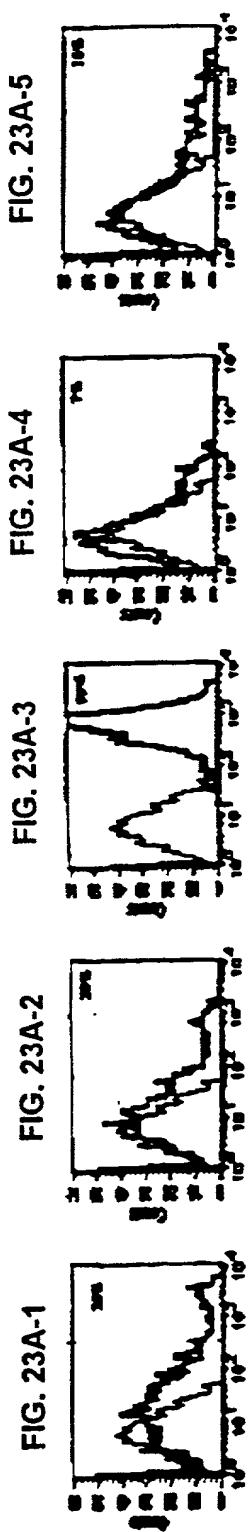
WO 00/76320

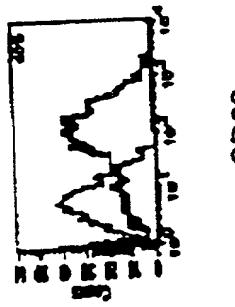
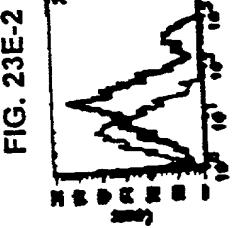
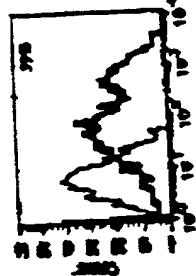
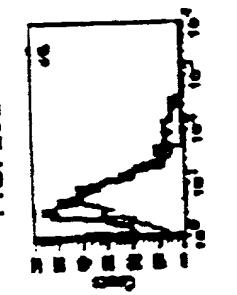
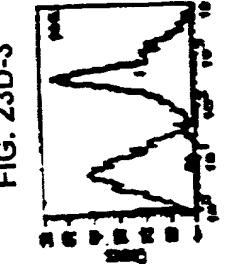
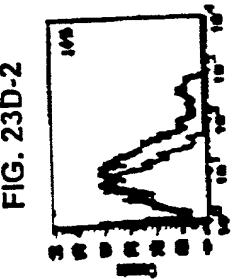
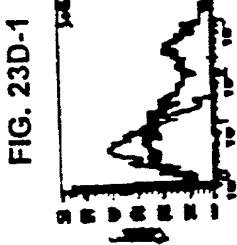
PCT/US00/16594

26/44

FIG. 22



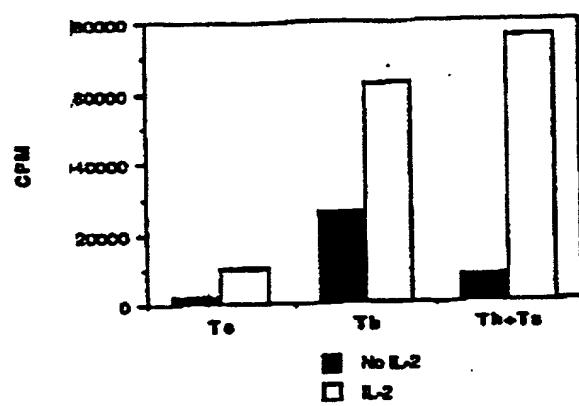




CD54 CD58 CD40 CD80 CD86

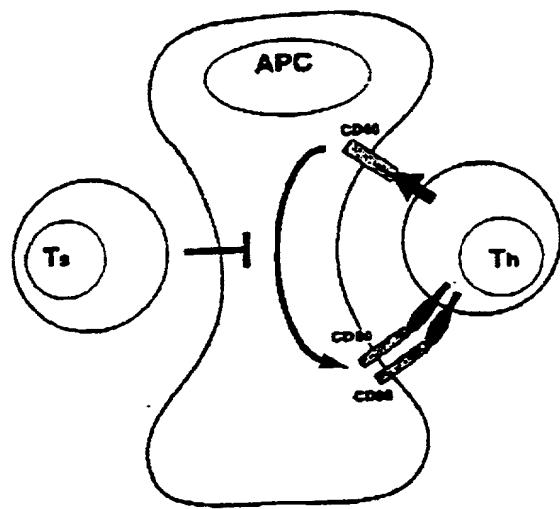
29/44

FIG. 24



30/44

FIG. 25



10/018677

WO 00/76320

PCT/US00/16594

31/44

FIG. 26

HLA A, B and DR Antigen Values and Split Equivalences

A LOCUS	EQUIVALENT	B LOCUS	EQUIVALENT	DR LOCUS	EQUIVALENT
1	1	5	5	1	1
2	2	7	7	2	2,15,16
3	3	8	8	3	3,17,18
9	9	12	12	4	4
10	10,66	13	13	5	5,11,12
11	11	14	14,64,65	6	6,13,14
19	19,74	15	15,75,76,77	7	7
23	23	16	16	8	8
24	24	17	17,88	9	9
25	25	18	18	10	10
26	26	21	21	11	11,5
28	28,58,69	22	22,54,65,66	12	12,5
29	29	27	27	13	13,5
30	30	35	35	14	14,5
31	31	37	37	15	15,2
32	32	38	38	16	16,2
33	33	39	39	17	17,3
34	34	40	40,51	18	18,3
35	35	41	41	51	51
43	43	42	42	52	52
65	65,10	44	44	53	53
68	68,28	45	45	103	103
69	69,23	46	46	1403	1403
74	74,19	47	47	1404	1404
80	80	48	48	99	99
- 203	203	49	49		
- 210	210	50	50		
- 2403	2403	51	51		
-- 99	99	52	52		
		53	53		
		54	54,22		
		55	55,22		
		56	56,22		
		57	57		
		58	58,17		
		59	59		
		60	60		
		61	61,40		
		62	62		
		63	63		
		64	64,14		
		65	65,14		
		67	67		
		70	70,71,72		
		71	71,70		
		72	72,70		
		73	73		
		75	75,15		
		76	76,15		
		77	77,15		
		- 703	703		
		- 3901	3901		
		- 3902	3902		
		- 4005	4005		
		- 5102	5102		
		- 5103	5103		
		- 7801	7801		
		- 8101	8101		
		-- 99	99		

-- Code 99 means not tested

32/44

FIG. 27A

DRB Protein Sequences - 20th March 1998 - SGE Marsh ANRI

1 10 20 30 40 50 60 70 80 90 100

DRB1*0101
 GDTRPRFLWQLKFECHFFNGTERVRLLERCIYNQEEESVRFDSDVGEYRAVTTELGRPDAEYWNSQK
 DLLEQRRAAVDTYCRHNYGVGESFTVQRRVEPKVTVY

DRB1*01021 ——————AV—————
 DRB1*01022 *****—————
 AV*****—————
 DRB1*0103 ——————I-DE—————
 DRB1*0104 ——————N—V—————
 DRB1*15011 —————P-R————F-D-YF————F————I-A————V————Q————
 —
 DRB1*15012 *****—P-R————F-D-YF————F————I-A————V————
 *****—————
 DRB1*15021 —————P-R————F-D-YF————F————I-A————Q————
 —
 DRB1*15022 *****—————F-D-YF————F————I-A————
 *****—————
 DRB1*15023 *****—P-R————F-D-YF————F————I-A————
 *****—————
 DRB1*1503 —————P-R————F-D-HF————F————I-A————V————Q————

 DRB1*1504 *****—P-R————F-D-YF————F————F-A————V————
 *****—————
 DRB1*1505 *****—P-R————F-D-YF————F————A————V————
 *****—————
 DRB1*1506 *****—P-R————F-D-YF————F-A————I-A————V————
 *****—————
 DRB1*16011 —————P-R————F-D-YF————F-D————Q————
 —
 DRB1*16012 *****—P-R————F-D-YF————F-D————
 *****—————
 DRB1*16021 —————P-R————F-D-YF————D————Q————
 DRB1*16022 *****—P-R————F-D-YF————D————
 *****—————
 DRB1*1603 —————P-R————F-D-YF————F-D-A————Q————
 —
 DRB1*1604 *****—P-R————F-D-YF————F-D-L————
 *****—————
 DRB1*1605 *****—P-R————F-D-YF————I-D————
 *****—————
 DRB1*1607 *****—P-R————FPD-YF————I-D————
 *****—————
 DRB1*1608 *****—P-R————F-D-YF—N————F————
 *****—————
 DRB1*03011 —————EYSTS————Y-D-YFH—N————F————K-GR-N————V————
 —H————
 DRB1*03012 *****—EYSTS————Y-D-YFH—N————F————K-GR-N————V————
 *****—————

33/44

FIG. 27B

DRB1*03021 ——EYSTS——F-YFH—N————K-GR-N————
 H—
 DRB1*03022 ——EYSTS——F-YFH—N————K-GR-N————
 H—**
 DRB1*0303 *****YSTS——F-YFH—N————K-GR-N—V—

 DRB1*0304 *****-EYSTS——Y-D-YFH————F————K-GR-N—V—

 DRB1*0305 *****—EYSTS——Y-D-YFH—N——F————K-GR-N————

 DRB1*0306 *****—EYSTS——Y-D-YFH—N————K-GR-N—V—

 DRB1*0307 ——EYSTS——F-D-YFH—N——F————K-GR-N—V—
 H—
 DRB1*0308 ——EYSTS——Y-D-YFH—N——F——E————K-GR-N—V—
 H—
 DRB1*0309 *****-EYSTS——Y-D-YFH-R-N——F————K-GR-N————

 DRB1*0310 ——EYSTS——Y-D-YFH—N——F——A-H————K-GR-N—V—
 H—
 DRB1*0311 *****—EYSTS——Y-D-YFH—N——F————K-GQ-N—V—

 DRB1*04011 ——E-V-H————F-D-YF-H—Y————K————Y—
 E—
 DRB1*04012 *****—E-V-H————F-D-YF-H—Y————K————

 DRB1*0402 ——E-V-H————F-D-YF-H—Y————I-DE————V—

 DRB1*0403 ——E-V-H————F-D-YF-H—Y————E————V—Y—
 E—
 DRB1*0404 ——E-V-H————F-D-YF-H—Y————V—Y—
 E—
 DRB1*04051 ——E-V-H————F-D-YF-H—Y————S————

 DRB1*04052 *****—E-V-H————F-D-YF-H—Y————S————

 DRB1*0406 ——E-V-H————F-D-YF-H————E————V—Y—
 E—
 DRB1*0407 ——E-V-H————F-D-YF-H—Y————E————

 DRB1*0408 *****—E-V-H————F-D-YF-H—Y————

 DRB1*0409 *****—E-V-H————F-D-YF-H—Y————S————K—

 DRB1*0410 *****—E-V-H————F-D-YF-H—Y————S————V—

 DRB1*0411 ——E-V-H————F-D-YF-H—Y————S————E————V—Y—
 E—
 DRB1*0412 *****—E-V-H————F-D-YF-H—Y————S————I-D-L————V—

 DRB1*0413 *****—H————F-D-YF-H—Y————K————V—

 DRB1*0414 *****—F-D-YF-H—Y————I-DE————

34/44

FIG. 27C

DRB1*0415 *****—E-V-H—F-D-YF-H—Y—E—F-D—V—

DRB1*0416 *****—F-D-YF-H—Y—Q—K—

DRB1*0417 *****—F-D-YF-H—Y—S—E—

DRB1*0418 *****—F-D-YF-H—Y—I-D-L—V—

DRB1*0419 *****H—F-D-YF-H—

DRB1*0420 *****—F-D-YF-H—E—

DRB1*0421 *****E-V-H—F-D-YF-H—K—

DRB1*0422 *****E-V-H—F-D-YF-H—Y—K—GR—N—V—

DRB1*0423 *****—E-V-H—F-D-YF-H—Y—V—R—

DRB1*0424 *****—E-V-H—F-D-YF-H—Y—S—R—

DRB1*0425 *****—E-V-H—F-D-YF-H—Y—F—D—L—V—

DRB1*0426 *****—E-V-H—F-D-YF-H—Y—T—K—

DRB1*0427 *****—E-V-H—F-D-YF-H—Y—E—AV—

DRB1*11011 —EYSTS—F-D-YF—Y—F—E—F—D—
H—
DRB1*11012 —EYSTS—F-D-YF—Y—F—E—F—D—
H—*
DRB1*11013 *****—EYSTS—F-D-YF—Y—F—E—F—D—

DRB1*1102 —EYSTS—F-D-YF—Y—F—E—I—DE—V—
H—
DRB1*1103 —EYSTS—F-D-YF—Y—F—E—F—DE—V—
H—
DRB1*11041 —EYSTS—F-D-YF—Y—F—E—F—D—V—

DRB1*11042 —EYSTS—F-D-YF—Y—F—E—F—D—V—
H—
DRB1*1105 ****—EYSTG—F-D-YF—Y—F—E—F—D—

DRB1*1106 *****—EYSTS—F-D-YF—Y—F—E—F—D—AV—

DRB1*1107 *****—EYSTS—F-D-YF—Y—F—E—K—GR—N—V—

DRB1*11081 *****—S—F-D-YF—Y—F—E—D—

DRB1*11082 *****—S—F-D-YF—Y—F—E—D—

DRB1*1109 *****—F-D-YFH—N—F—E—F—D—

DRB1*1110 *****—F-D-YFH—F—F—E—F—D—

35/44

FIG. 27D

DRB1*1111 *****S-----F-D-YF---Y---F---E---F-DE-----

DRB1*1112 *****-----F-D-YF---F---F---E---F-D-----

DRB1*1113 -----EYSTS-----F-D-YFH---F---F---E---R---V-----

DRB1*1114 -----EYSTS-----F-D-YF---Y---F---E---I-DE-----

DRB1*1115 -----EYSTS-----F-D-YF---DL---F---E---F-D-----
H---
DRB1*1116 *****-EYSTS-----F-D-YFH---N---F---E---I-DE-----V-----

DRB1*1117 -----EYSTS-----F-D-YFH---F---E---R-E---V-----
H---
DRB1*1118 *****-EYSTS-----F-D-YF---Y---F---E---I-D---V-----

DRB1*1119 *****-EYSTS-----F-D-YF---Y---F---E---I-D-----

DRB1*1120 *****-EYSTS-----F-D-YFH---N---F---E---I-DE-----

DRB1*1121 *****-EYSTS-----F-D-YF---Y---F---E---I-DE---AV-

DRB1*1122 *****-E-V-H-----F-D-YF---Y---F---E---F-D-----

DRB1*1123 ****-EYSTS-----F-D-YF---Y---F---E---F-D-L-----

DRB1*1124 *****-EYSTS-----F-D-YF---D---F---E---F-D-----

DRB1*1125 *****-EYSTS-----F-D-YF---Y---F---E---F-D-L---V-----

DRB1*1126 *****-EYSTS-----F-D-YF---Y---F---E-----

DRB1*1127 *****-EYSTS-----F-D-YF---Y---F---E---F-D---N-----

DRB1*1128 *****-EYSTS-----F-D-YF---N---F---E---F-D-----

DRB1*1129 *****-EYSTS-----F-D-YF-----F---E---F-D-----

DRB1*1130 *****-EL-S-----F-D-YF---Y---F---E---F-D-----

DRB1*1131 -----EYSTS-----F-D-YF---Y---F---E-H---I-D-----
H---
DRB1*1201 -----EYSTG-Y-----HFH---LL---F---V-S---I-D-----AV-
H---
DRB1*12021 *****-EYSTG-Y-----HFH---LL---F---V-S---F-D-----
AV*****
DRB1*12022 *****-EYSTG-Y-----HFH---LL---F---V-S---F-D-----AV-

DRB1*12032 *****-EYSTG-Y-----HFH---LL---F---V-S---I-D-----V-----

DRB1*1204 *****-EYSTG-Y-----HFH---LL---F---E---I-D-----
AV*****
DRB1*1205 *****-EYSTG-Y-----HFH---FL---F---V-S---I-D-----AV-

36/44

FIG. 27E

DRB1*1301 ——EYSTS——F-D-YFH—N—F——I-DE——V
 H——
 DRB1*1302 ——EYSTS——F-D-YFH—N—F——I-DE——
 H——
 DRB1*13031 ——EYSTS——F-D-YF—Y——S——I-DK——
 H—*
 DRB1*13032 *****—EYSTS——F-D-YF—Y——S——I-DK——

 DRB1*1304 ——EYSTS——F-D-YF—Y——F——S——I-DE——V
 H—*
 DRB1*1305 *****—EYSTS——F-D-YFH—N—F——F-D——

 DRB1*1306 *****—F-D-YFH—N—F——I-D——V——

 DRB1*13071 *****—EYSTS——F-D-YF—Y——F-D——

 DRB1*1308 *****—EYSTS——F-D-YFH—F——I-DE——V——

 DRB1*1309 *****—EYSTS——F-D-YFH—N—F——I-A——V——

 DRB1*1310 *****—EYSTS——F-D-YFH—N—F——I-DK——V——

 DRB1*1311 *****—EYSTS——F-D-YF—Y——F——F-D——V——

 DRB1*1312 *****—EYSTS——F-D-YF—Y——S——I-D——

 DRB1*1313 *****—EYSTS——F-D-YF—Y——S——I-D-L——

 DRB1*1314 *****—TS——F-D-YF—Y——F——F-D——

 DRB1*1315 *****—EYSTS——F-YFH—N—F——I-DE——V——

 DRB1*1316 *****—EYSTS——F-D-YFH—N—F——I-DE——D——

 DRB1*1317 ——EYSTG—Y——F-D-YF—Y——F——I-DE——V——
 H——
 DRB1*1318 *****—EYSTS——F-D-YFH—N—F——F-D-L——V——

 DRB1*1319 ——EYSTS——F-YFH—F——I-DE——V——H——
 ——
 DRB1*1320 *****—EYSTS——F-D-YFH—N—F——DE——V——

 DRB1*1321 ——EYSTS——F-D-YF—Y——F——S——F-D——H——
 ——
 DRB1*1322 ****—EYSTS——F-D-YF—Y——F——I-DE——V——

 DRB1*1323 ****—EYSTS——F-D-YF—Y——F——I-DE——

 DRB1*1324 ****—EYSTS——F-D-YF—Y——F——F-DE——V——

 DRB1*1325 ****—EYSTS——F-D-YF—Y——F——D——

 DRB1*1326 ****—EYSTS——F-YFH—N——F-D——

WO 00/76320

37/44

FIG. 27F

DRB1*1327 *****-EYSTS-----Y-D-YFH-N-F-----I-DE-----V-----
 *****-
 DRB1*1328 *****-EYSTS-----F-D-YFH-N-F-----I-DE-----R-V-----
 *****-
 DRB1*1329 *****-EYSTS-----F-D-YFH-N-F-----DE-----
 *****-
 DRB1*1330 *****-EYSTS-----F-D-YF-Y-F-S-----I-D-----
 *****-
 DRB1*1331 *****-EYSTS-----F-D-YFH-N-F-V-----I-DE-----
 *****-
 DRB1*1332 *****-EYSTS-----F-D-YFH-N-S-----I-DE-----V-----
 *****-
 DRB1*1333 *****-EYSTS-----F-D-YF-Y-S-----I-DK-N-----
 *****-
 DRB1*1401 ---EYSTS-----F-D-YFH-F-----A-H-----R-E-----V-----
 *****-
 DRB1*1402 ---EYSTS-----F-YFH-N-----
 *****-
 DRB1*1403 ---EYSTS-----F-YFH-N-----D-L-----
 *****-
 DRB1*1404 *****-EYSTG-Y-----F-D-YFH-F-----A-H-----R-E-----V-----
 *****-
 DRB1*1405 *****-EYSTS-Q-----F-D-YFH-F-----R-E-----V-----
 *****-
 DRB1*1406 *****-EYSTS-----F-YFH-N-----V-----
 *****-
 DRB1*1407 *****-EYSTS-----F-D-YFH-F-----A-H-----R-E-----
 *****-
 DRB1*1408 *****-EYSTS-----F-D-YFH-F-----H-----R-E-----V-----
 *****-
 DRB1*1409 *****-EYSTS-----F-D-YFH-N-----
 *****-
 DRB1*1410 *****-E-V-H-----F-D-YFH-F-----A-H-----R-E-----V-----
 *****-
 DRB1*1411 *****-EYSTG-Y-----F-D-YFH-F-----E-----R-E-----
 V*****-
 DRB1*1412 *****-S-----F-YFH-N-----D-L-----
 V*****-
 DRB1*1413 *****-EYSTS-----F-YFH-N-----S-----
 *****-
 DRB1*1414 *****-EYSTS-----F-D-YFH-F-----R-E-----
 *****-
 DRB1*1415 *****-STG-Y-----F-D-YFH-F-----F-D-L-----V-----
 *****-
 DRB1*1416 *****-EYSTS-----F-D-YFH-F-----A-H-----I-DE-----V-----
 *****-
 DRB1*1417 *****-EYSTS-----F-D-YFH-N-F-----V-----
 *****-
 DRB1*1418 *****-EYSTS-----F-YFH-N-----R-E-----V-----
 *****-
 DRB1*1419 ---EYSTS-----F-YFH-N-----K-----
 *****-
 DRB1*1420 *****-EYSTS-----F-YFH-F-----V-----

10/018677

WO 00/76320

PCT/US00/16594

38/44

FIG. 27G

DRB1*1421 *****-EYSTS—F-D-YFH—N—F—K—V

 DRB1*1422 *****-EYSTS—F-D-YFH—F—A-H—F-D

 DRB1*1423 *****-EYSTS—F-D-YFH—F—R-E—V

 DRB1*1424 *****-EYSTS—F-YFH—N—I-A
 H—
 DRB1*1425 *****-EYSTS—F-D-YF—Y—A-H—F-D

 DRB1*1426 *****-EYSTS—QF-D-YFH—F—A-H—R-E—V
 —*****
 DRB1*1427 *****-EYSTS—F-YFH—N—F-D-L

 DRB1*1428 *****-EYSTG-Y—F-D-YFH—F—A-H—R-E
 AV*****
 DRB1*1429 *****-EYSTS—F-YFH—N—AV

 DRB1*1430 *****-EYSTS—F-D-YFH—N—F

 DRB1*1431 *****-EYSTG-Y—F-D-YFH—F—A-H—R—V

 DRB1*0701 —Q—G-YK—QF—LF—F—V-S—I-D-GQ—V
 H-E—
 DRB1*0703 *****-G-YK—QF—SLF—F—V-S—I-D-GQ—V

 DRB1*0801 —EYSTG-Y—F-D-YF—Y—S—F-D-L

 DRB1*08021 —EYSTG-Y—F-D-YF—Y—F-D-L
 H—
 DRB1*08022 *****-EYSTG-Y—F-D-YF—Y—F-D-L
 H—
 DRB1*08032 —EYSTG-Y—F-D-YF—Y—S—I-D-L
 H—
 DRB1*08041 —EYSTG-Y—F-D-YF—Y—F-D-L—V
 H-*
 DRB1*08042 *****—F-D-YF—Y—F-D-L—V

 DRB1*08043 *****-EYSTG-Y—F-D-YF—Y—F-D-L—V

 DRB1*0805 *****-EYSTG-Y—F-D-YF—Y—S—F-D

 DRB1*0806 *****-EYSTG-Y—F-D-YF—Y—S—F-D-L—V

 DRB1*0807 *****-EYSTG-Y—F-D-YF—Y—V—F-D-L

 DRB1*0808 *****-EYSTG-Y—F-D-YF—Y—A-H—F-D-L

 DRB1*0809 *****—Y—F-D-YFH—F—F-D-L

 DRB1*0810 *****-EYSTG-Y—F-D-YF—Y—S—I-D-L—V

 DRB1*0811 *****-EYSTG-Y—F-D-YF—Y—A—F-D-L

FIG. 27H

DRB1*0812 *****-EYSTG-Y-F-D-YF-Y-S-I-D-L-AV-

DRB1*0813 *****-EYSTG-Y-F-D-YF-Y-D-L-

DRB1*0814 *****-EYSRG-Y-F-D-YF-Y-S-I-D-L-

DRB1*0815 *****-EYSTG-Y-F-D-YF-Y-H-I-D-L-

DRB1*0816 *****-EYSTG-Y-F-D-YF-D-S-F-D-L-

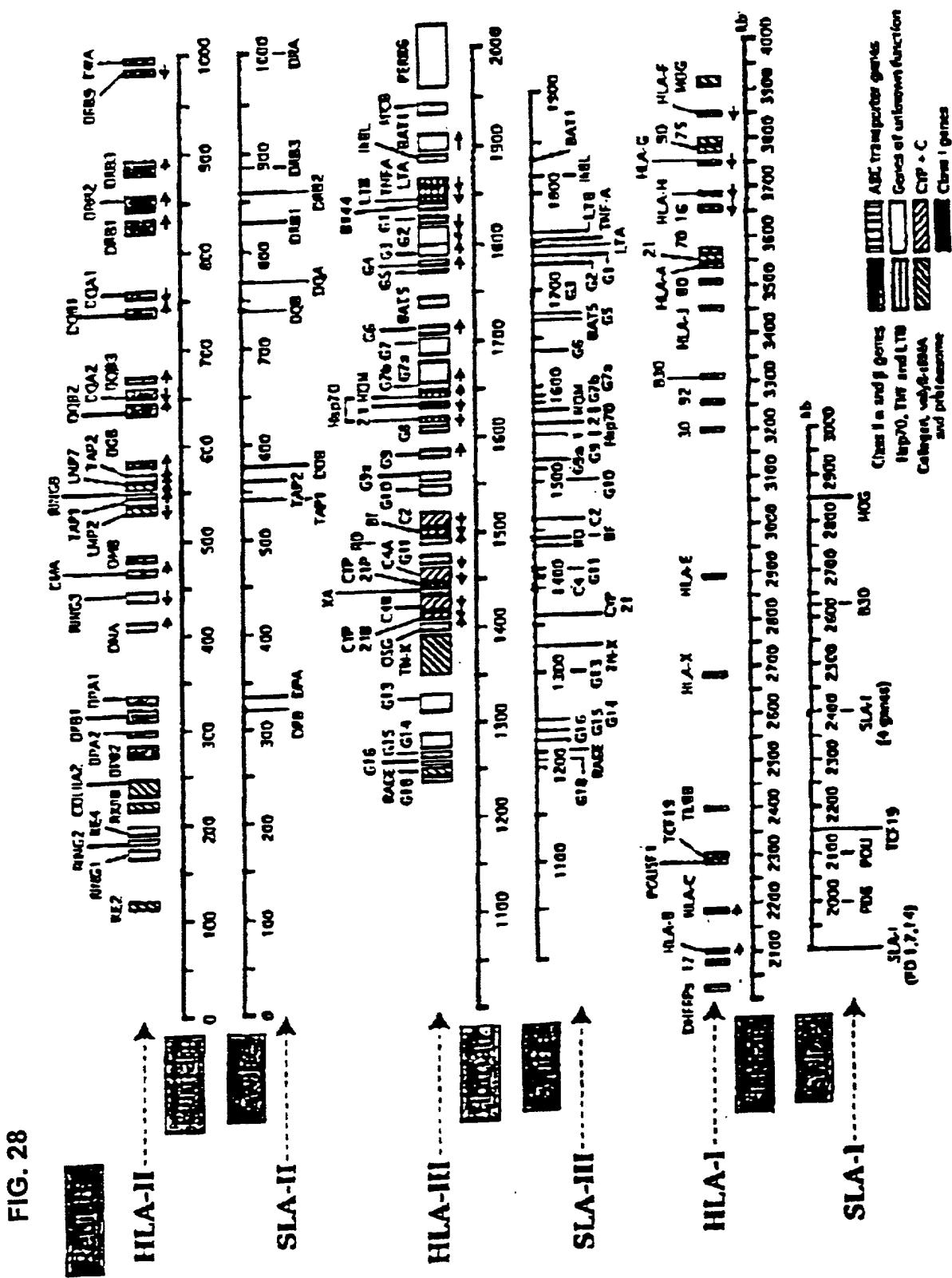
DRB1*0817 *****-EYSTG-Y-F-D-YF-Y-F-S-F-D-L-

DRB1*0818 *****-EYSTG-Y-F-D-YF-Y-S-I-D-

DRB1*0819 *****-EYSTG-Y-F-D-YF-Y-I-I-D-L-

DRB1*09012 -Q-K-D-Y-H-G-N-V-S-F-R-E-V-
H-E-
DRB1*1001 -EEV-RVH-YA-Y-R-Q-
—

40/44



197010677

WO 00/76320

PCT/US00/16594

41/44

FIG. 29

	1		50
Sladra-0102	-----	-----	-----
Sladra-0202	-----	-----	-----
Sladra-0203	-----	-----	-----
Sladra-0101	-----	-----	-----
Sladra-02011	-----	-----	-----
Sladra-02012	-----	-----	-----
Consensus	VENHVIIQAE FYLSPDKSGE FMFDGDGDEI FHVDMEKRET VWRLEEFHF		
	51		100
Sladra-0102	-----	-----	-----
Sladra-0202	-----	-----	-----
Sladra-0203	-----	-----	-----
Sladra-0101	-----	-----	-----
Sladra-02011	-----	-----	-----
Sladra-02012	-----	-----	-----
Consensus	ASFEAQGALA NIAVOKANLE ILIKRSNNTP NTNVPPEVTV LSDKPVELGE		
	101		150
Sladra-0102	-----	-----	-----
Sladra-0202	-----	-----	-----
Sladra-0203	-----	-----	-----
Sladra-0101	-----	-----	-----
Sladra-02011	-----	-----	-----
Sladra-02012	-----	-----	-----
Consensus	PNILICFIDK FSPPVVNVTW LRNGSPVTRG VSETVFLPRE DHLFRKFHYL		
	151		200
Sladra-0102	-----	-----	-----
Sladra-0202	-----	-----	-----
Sladra-0203	-----	-----	-----
Sladra-0101	-----	-----	-----
Sladra-02011	-----	-----	-----
Sladra-02012	-----	-----	-----
Consensus	PFMPSTEDVY DCQVEHWGLD KPLLKHWEFE AQTPLPETTE NTVCALGLIV		
	201		228
Sladra-0102	-----	-----	-----
Sladra-0202	-----	-----	-----
Sladra-0203	-----	-----	-----
Sladra-0101	-----	-----	-----
Sladra-02011	-----	-----	-----
Sladra-02012	-----	-----	-----
Consensus	ALVGIIIVGTV LIIKGVRKGN ATERRGPL		

Group 01 has a leucine at residue 72 and Group 02 has a methionine.
 No other polymorphisms have been found in the alpha 1 domain.

Amino Acid Sequences of SLA DRA Alleles

42/44

FIG. 30

	1	21	41	50
Sladrb-T	--iaq--ffm q-s	y-qky l	-----	1-f-----
Sladrb-N	-----f- g-a	d-y f-d-y	-----	f-e-----
Sladrb-M	-----f- g	q-----	-----	-----
Sladrb-Z	-----y-	-----	-----y	-----
Sladrb-AD	-----	-----	-----l	-----
Sladrb-C	--i-----q	-----q-n c-----y	-----	-----
Sladrb-WX	-----v-h-r	-----l-d-v f-----	-----	1-f-----
Sladrb-Y	--i-----ffm g-s	-----y-lky l	-----	1-----e
Consensus	RDTPPHFLHL LKFECHFFNG TERVRLLERQ YYNGEEFVRF DSDVGEYRAV			
	51	64	81	100
Sladrb-T	-----	m k-v	-----	-----
Sladrb-N	-----f-----	fm k-v	-----e-e-----	r-----
Sladrb-M	-----n y	-----	-----ts-----	r-----
Sladrb-Z	-----v-d	-----	-----ts-----	r-----
Sladrb-AD	-----d	-----	-----	-----
Sladrb-C	-----r-----	a-----	-----	-----
Sladrb-WX	-----	i-d s-s-i	-----	-----
Sladrb-Y	-----	-----ek-----	gvs-s-----	-----
Consensus	TELGRPDAKY WNSQKDLEQ RRAEVDTYCR HNYRILDTEFL VPRRAEPTVT			
	101			150
Sladrb-T	-----	-----	-----	-----
Sladrb-N	-----	-----	-----	-----
Sladrb-M	-----	-----	-----	-----
Sladrb-Z	-----	-----	-----	-----
Sladrb-AD	-----	-----	-----	-----
Sladrb-C	-----	-----	-----	-----
Sladrb-WX	-----	-----	-----	-----
Sladrb-Y	-----	-----	-----	-----
Consensus	VYPAKTQPLQ HENLLVCSVY GFYPGHVEVR WFRNGOEAAA GVVSTGLIPN			
	151			200
Sladrb-T	-----	-----	-----	-----
Sladrb-N	-----	t-----	-----	-----
Sladrb-M	-----	-----	-----	-----
Sladrb-Z	-----	-----	-----	-----
Sladrb-AD	-----	-----	-----	-----
Sladrb-C	-----	-----	-----	-----
Sladrb-WX	-----	-----	-----	-----
Sladrb-Y	-----	-----	-----	-----
Consensus	GDWTFQTMVM LETVPQSGEV YSCRVEHPSL TSPVTVEWRA RSESAQGKMM			
	201		237	
Sladrb-T	--v-----	-----	-----	-----
Sladrb-N	-----	-----	-----	-----
Sladrb-M	-----	-----	-----	-----
Sladrb-Z	--v-----	-----	-----	-----
Sladrb-AD	-----	-----	-----	-----
Sladrb-C	--v-----	-----	-----	-----
Sladrb-WX	-----	-----	-----	-----
Sladrb-Y	--v-----	-----	-----	-----
Consensus	SGIGGFVLGL LFVAVGLFIY FKNQKGRPAL QPTGLLS			

Amino Acid Sequence of SLA-DRB Alleles

43/44

FIG. 31

		50
Sladqa-02021	-----	g
Sladqa-02022	-----	g
Sladqa-02023	-----	-----
Sladqa-0201	-----	r q
Sladqa-0101	-----	r f q
Sladqa-0103	-----	f
Sladqa-0102	-----	-----
Consensus	EDIAADHVAS YGLNVYQSYG PSGYYTHEFD GDEEFYVDLE KKETVWQLPL	
		100
Sladqa-02021	-----r-----	-----
Sladqa-02022	-----r-----	-----
Sladqa-02023	-----r-----	-----
Sladqa-0201	-----	l vt k s
Sladqa-0101	---e---	l vt k s
Sladqa-0103	---e---	l vt k s
Sladqa-0102	---e---	l vt k s
Consensus	FSKFTSFDPQ GALRNIATAK HNLNLIKRS NNAAVNQVP EVTVEPKSPV	
		150
Sladqa-02021	-----	-----
Sladqa-02022	-----	-----
Sladqa-02023	-----	-----
Sladqa-0201	-----	.k
Sladqa-0101	i-----s-----	.k
Sladqa-0103	i-----s-----	.k
Sladqa-0102	i-----s-----	.k
Consensus	MLGQPNTLIC HVDNIFPPVI NITWLKNHGS VTEGFSETSF LSKNDHSFLK	
		200
Sladqa-02021	-----	-----
Sladqa-02022	-----	-----
Sladqa-02023	-----	-----
Sladqa-0201	-----	-----
Sladqa-0101	-----	-----
Sladqa-0103	-----	-----
Sladqa-0102	-----	-----
Consensus	ISYLTFLPSD DDFYDCKVEH WGLDKPLLKH WEPEIPAPMS ELTETVVCAL	
		232
Sladqa-02021	-----	-----
Sladqa-02022	-----	-----
Sladqa-02023	-----	-----
Sladqa-0201	-----	-----
Sladqa-0101	-----	-----
Sladqa-0103	-----	-----
Sladqa-0102	-----	-----
Consensus	GLIVGLVGIV VGTVFIIQGL RSGGPSRHQG SL	

Group 01 is 231 amino acids (deletion at 132) and Group 02 is 232 amino acids

Amino Acid Sequences of SLA-DQA Alleles

10/018677

PCT/US00/16594

WO 00/76320

44/44

FIG. 32

	1	50
Sladqb-D	v - g - y - ws-d - fl - m - y -	
Sladqb-D2	f - g - y - ws-d - fl - m - y -	
Sladqb-XA	- - - - - g - wv -	
Sladqb-Z	- - - - - y - i - wv - r -	
Sladqb-W	- - - - - - - i-t - ya - y -	
Sladqb-T	- - - - - - - llt - n - y -	
Sladqb-Y	- - - - - g - h-t - - -	
Sladqb-C	- - - f - g - y - g - l -	
Consensus	GRDSPQDFVY QFKFECYFFN GTQRVR-VAR YIYNQEEHVR FDSDVGEGRA	
	51	100
Sladqb-D	- - - l - ea - - - - -	
Sladqb-D2	- - - l - ea - - - - -	
Sladqb-XA	- t - - - - v -	
Sladqb-Z	- - - e - - - - -	
Sladqb-W	- a - s - i - t -	
Sladqb-T	- - - - - t -	
Sladqb-Y	- - - f - t -	
Sladqb-C	- e - s - s - m - v - r -	
Consensus	VTPLGRPDAD YWNGQKDVL E QKRAELDTV C KHN YQIEEGT TLQRRVQPTV	
	101	150
Sladqb-D	- - - - - - - - - - -	
Sladqb-D2	- - - - - - - - - - -	
Sladqb-XA	- - - - - - - - - - -	
Sladqb-Z	- - - - - - - - - - -	
Sladqb-W	- - - - - - - - - - -	
Sladqb-T	- - - - - - - - - - -	
Sladqb-Y	- - - - - - - - - - -	
Sladqb-C	- - - - - - - - - - -	
Consensus	TISPSKAЕAL NHHNLLVCAV TDFYPSQVKV QWFRNGQEET AGVVSTPLIR	
	151	200
Sladqb-D	- - - - - - - - - - -	
Sladqb-D2	- - - - - - - - - - -	
Sladqb-XA	- - - - - - - - - - -	
Sladqb-Z	- - - - - - - - - - -	
Sladqb-W	- - - - - - - - - - -	
Sladqb-T	- - - - - - - - - - -	
Sladqb-Y	- - - - - - - - - - -	
Sladqb-C	- - - - - - - - - - -	
Consensus	NGDWTYQVLV MLEMNLQRGD VYTCRVEHSS LQSPILVEWR AQSESAQSKM	
	201	230
Sladqb-D	- - - - - - - - - - -	
Sladqb-D2	- - - - - - - - - - -	
Sladqb-XA	- - - - - - - - - - -	
Sladqb-Z	- - - - - - - - - - -	
Sladqb-W	- - - - - - - - - - -	
Sladqb-T	- - - - - - - - - - -	
Sladqb-Y	- - - - - - - - - - -	
Sladqb-C	- - - - - - - - - - -	
Consensus	LSGVGGFVLG LIFLGLGLFI RHRSQKGLVR	

Amino Acid Sequences of SLA-DQB Alleles

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GENERATION OF ANTIGEN SPECIFIC T SUPPRESSOR CELLS
FOR TREATMENT OF REJECTION

*the specification of which:
(check one)*

is attached hereto.

was filed on December 14, 2001 as

Application Serial No. 10/018,677

*and was amended _____
(if applicable)*

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed:

Prior Foreign Application(s)**Priority Claimed**

<u>Number</u>	<u>Country</u>	<u>Filing Date</u>	<u>Yes</u>	<u>No</u>
PCT/US00/16594	PCT	15 June 2000	X	—
_____	_____	_____	—	—
_____	_____	_____	—	—
_____	_____	_____	—	—
_____	_____	_____	—	—
_____	_____	_____	—	—

Declaration and Power of Attorney

Page 3

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

<u>Provisional Application No.</u>	<u>Filing Date</u>	<u>Status</u>
N/A		

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
09/333,809	June 15, 1999	Pending
PCT/US00/16594	June 15, 2000	Pending

And I hereby appoint

John P. White (Reg. No. 28,678); Christopher C. Dunham (Reg. No. 22,031); Norman H. Zivin (Reg. No. 25,385); Jay H. Maioli (Reg. No. 27,213); William E. Pelton (Reg. No. 25,702); Robert D. Katz (Reg. No. 30,747); Peter J. Philips (Reg. No. 29,691); Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No. 33,970); Roberto T. Maldonado (Reg. No. 38,232); Paul Teng (Reg. No. 40,837); Richard F. Jaworski (Reg. No. 33,515); Pedro C. Fernandez (Reg. No. 41,741); Gary J. Gershik (Reg. No. 39,992); Spencer H. Schneider (Reg. No. 45,923); Alan J. Morrison (Reg. No. 37,399); Alan D. Miller (Reg. No. 42,889); and Frank Bruno (Reg. No. 46,583)

and each of them, all co^o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Declaration and Power of Attorney

Page 3

Please address all communications, and direct all telephone calls, regarding this application to:

John P. White

28,678

Reg. No.

**Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
Tel. (212) 278-0400**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**Full name of sole or
first joint inventor** Nicole Suciu-Foca

Inventor's signature Nicole Suciu-Foca

Citizenship United States of America **Date of signature** 1/30/2002

Residence 120 Central Park South, Apt. 9A, New York, New York 10019

Post Office Address same as residence address NY

**Full name of joint
inventor (if any)** Raffaello Cortesini

Inventor's signature Raffaello Cortesini

Citizenship Italy **Date of signature** 1/30/2002

Residence 120 Central Park South, Apt. 9A, New York, New York 10019

Post Office Address same as residence address NY

**Full name of joint
inventor (if any)** Zhuoru Liu

Inventor's signature Zhuoru Liu

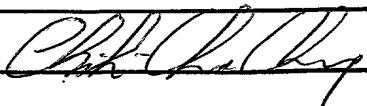
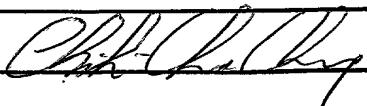
Citizenship United States of America **Date of signature** 1/30/2002 NY

Residence 100 Haven Avenue, Apt. 9C, New York, New York 10032

Post Office Address same as residence address

Y.H.
Declaration and Power of Attorney

Page 4

**Full name of joint
inventor (if any)** Chih-Chao Chang 
Inventor's signature 
Citizenship United States of America **Date of signature** 1/30/02
Residence 555 Central Park Avenue, Apt. 228, Scarsdale, New York 10583
Post Office Address same as residence address NY

**Full name of joint
inventor (if any)** _____
Inventor's signature _____
Citizenship _____ **Date of signature** _____
Residence _____
Post Office Address _____

**Full name of joint
inventor (if any)** _____
Inventor's signature _____
Citizenship _____ **Date of signature** _____
Residence _____
Post Office Address _____

Applicants: Nicole Suciu-Foca et al.

Serial No.: 10/018,677

Filed : December 14, 2001

Page : 2

C.F.R. §1.53(f). In compliance with 37 C.F.R. §1.497(a) and (b), the Declaration refers to the application's above-identified international application number and international filing date.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicant's undersigned attorney invites the Examiner to telephone the number provided below.

No fee, other than the enclosed surcharge of \$65.00 for late filing of the oath or declaration, is deemed necessary in connection with the filing of this Communication. If any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

05/20/2002 GFREY1 00000104 10018677

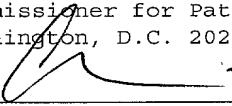
01 FC:254

65.00 OP

Respectfully submitted,



John P. White
Registration No. 28,678
Alan J. Morrison
Registration No. 37,399
Attorneys for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents Washington, D.C. 20231	
	5/7/02
Alan J. Morrison Reg. No. 37,399	Date